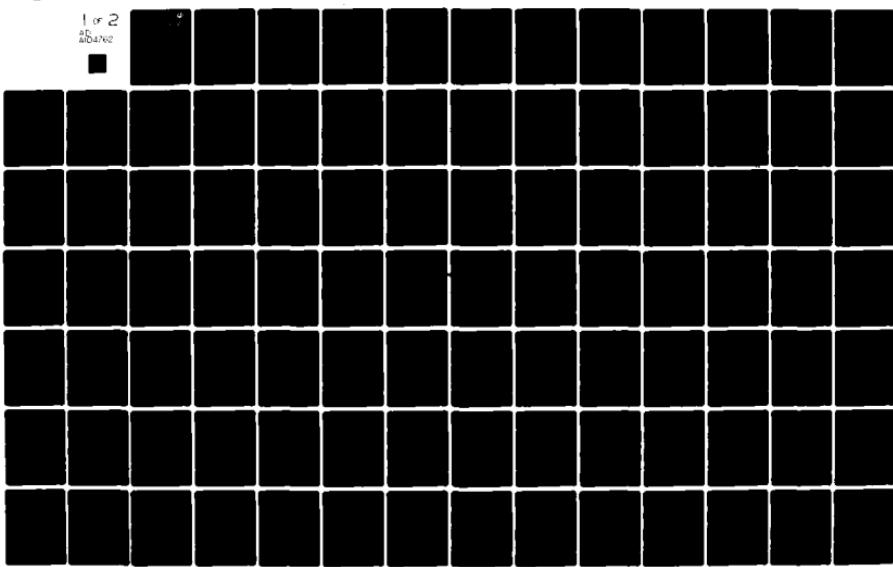


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# EVALUATION OF SHORT-TERM BIOASSAYS TO PREDICT FUNCTIONAL IMPAIRMENT

## SELECTED SHORT-TERM HEPATIC TOXICITY TESTS Final Report

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Ralph Wands  
Awadh Singh  
Lydia Thomas

October 1980

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The MITRE Corporation has been requested by the U.S. Army Medical Bioengineering Research and Development Laboratory to identify and evaluate short-term bioassays which have demonstrated ability to assess and predict impairment of the hepatic system resulting from short-term exposure to chemicals. This document reviews the literature on test procedures for determining toxic effects on the liver. The procedures are discussed in sections on morphology, function and biochemistry. Criteria for evaluating the procedures are given. A three-tiered testing system is		

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recommended for a chemical hepatotoxicity screening program.

This report is accompanied by a directory entitled Development of Hepatic Bioassays in Laboratory Animals: Directory of Institutions/Individuals.

The Directory catalogues the individuals and organizations currently engaged in hepatic bioassay utilization or development, and provides information concerning specific measurements performed, test systems employed, compounds tested, requirements for anesthesia, and the terminal nature of the test.)

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## EXECUTIVE SUMMARY

The Metrek Division of The MITRE Corporation, under contract to the United States Army Medical Bioengineering Research and Development Laboratory, is reviewing and recommending short-term tests in animals for evaluating and predicting the functional and/or morphological impairment produced by toxic substances. This document presents information on various tests that have been developed to detect liver damage. Recommendations are made for those tests which are suitable for use in a screening program.

The tests that measure the effects of xenobiotics on the liver are grouped into three sections or categories: morphology, functionality, biochemistry. The literature on the tests in each of these categories is reviewed and the tests evaluated for incorporation in a short-term screening program.

The morphologic techniques used to measure damage range from gross observation to electron microscopy. Gross observation of changes in the shape, size, color and consistency of the liver that occur as the result of the toxic effects of chemicals is necessary in early phases of screening chemical substances and may indicate the need for more detailed examination.

Microscopic examination permits an elaboration of the gross observations and may be necessary to provide a definitive description of liver damage. The very high magnification by the electron microscope reveals details, such as the dimensions of subcellular structures, which are useful for research purposes, for detecting early damage, or for an understanding of the mechanisms whereby toxic damage occurs.

Two general methods for assessing toxic effects on the secretory function of the liver are described. In the first, one of several dyes is injected intravenously and the rate of its disappearance from the blood stream is determined. In the second method, the rate of removal of a normal constituent, bilirubin, from the blood by the liver is monitored by determining serum bilirubin levels. Monitoring urobilinogen, a bilirubin metabolic product, in urine is less direct but effective in monitoring liver function.

The biochemical tests are grouped into two broad categories (1) direct measurements of the activity of specific serum enzymes and (2) measurements of substances related to liver synthesis, storage, detoxification and elimination. The hepatotoxicity of exogenous chemicals usually leads to changes in the permeability of the cell wall and

release of cellular components, including enzymes, into the blood stream. The extent and nature of liver damage may be indicated by measuring the activity of the enzymes released.

Damage may also be detected by measuring variations in the rates of metabolism of both endogenous and exogenous substances. As examples, the monitoring of the metabolic levels of carbohydrates, cholesterol, bile acids, serum metals, protein, blood-clotting factors and xenobiotics are described.

In addition to the biochemical and functional tests performed in vivo it is also possible to expose in vitro excised livers, liver slices, cell cultures or subcellular suspensions to xenobiotics, and to measure toxicity by monitoring various biochemical parameters.

The tests for liver damage are evaluated for incorporation in a short-term screening program according to their degree of conformance to the following criteria:

- state of development sufficient to give reproducible results in a screening program
- sensitivity sufficient to detect early subtle forms of damage to the system
- procedures and instrumentation sufficiently uninvolved to enable technicians with some additional training to perform the tests, and
- methods sufficiently brief so that each test can be completed within a few hours to several days.

In addition to these criteria, consideration has also been given to (1) the availability and cost of the animals used, and (2) the costs of the test procedures and equipment.

Those tests which satisfy these criteria have been sorted to form a three-tiered program for testing chemicals for their hepatotoxicity according to the following second set of criteria. These tests may be used with any mammalian species.

Level I of the tiered program consists of those tests which are simple, inexpensive, quick and sufficiently sensitive to provide a good indication of hepatic damage. Level II tests are more selective and sensitive than those in Level I and provide more information on the nature and mechanism of the toxic injury. Level III tests are those tests not included in Level I and II but which may be useful in determining the mechanism of the toxic injury.

The following three-tier, short-term testing series presents the tests recommended for inclusion in a chemical hepatotoxicity screening program.

Level I

Functional: Sulfobromophthalein (BSP) or Indocyanine (ICG)  
Clearance; Bilirubin Clearance (Plasma Bilirubin and Urine  
Urobilinogen); Benzoate/Hippuric Acid Excretion

Biochemical: Barbiturate Sleeping Time

Serum Enzymes: Glutamic-Oxalacetic Transaminase (GOT);  
Glutamic-Pyruvic Transaminase (GPT); Alkaline Phosphatase (ALP);  
Lactic Dehydrogenase (LDH)

Morphological: Gross Liver Pathology

Level II

Functional: Serum Cholesterol/Cholesterol Ester Ratio; Plasma Bile  
Acids; Biliary Transport Maximum (Tm)

Biochemical: Isolated Hepatocyte Suspensions or Monolayer Hepatocyte  
Cultures

Morphological: Light and Electron Microscopy

Level III

Functional: Radiolabeled Albumin or  $^{133}\text{Xe}$  Perfusion

Biochemical: In-Vitro Preparations (Other than those in Level II, e.g.,  
liver slices or isolated, perfused whole livers)

Morphological: Radioactive Colloid Imaging

## FOREWORD

The authors express their appreciation to Dr. Mary Henry, Project Officer of the U.S. Army Medical Bioengineering Research and Development Laboratory, for the support and guidance that she provided during the course of the project. The expert contributions by Herbert H. Cornish, Ph.D and Harold C. Grice, D.V.M., who submitted critical reviews of this report in its draft form, are gratefully acknowledged. We also wish to thank Kathleen Weston, M.D., for her expert contributions and technical support. Leadership and advice by Dr. Paul Clifford and Dr. Barbara Fuller throughout the course of the project have been of great value. The editorial and technical assistance by Ms. Lee Johnson and Ms. Yasuko Anglin, respectively, is sincerely appreciated.

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## 1.0 INTRODUCTION

The Metrek Division of The MITRE Corporation, under contract to the United States Army Medical Bioengineering Research and Development Laboratory, is reviewing and recommending short-term tests for evaluating the functional and/or morphological impairment produced by toxic substances in animal test systems. Effects in four organ systems--pulmonary, hepatic, renal, and cardiovascular--are being considered. This document presents information on the available tests for the hepatic system and recommends those tests which are suitable for use in a screening program.

A variety of tests have been developed to detect liver damage in humans. Some of these have been adapted for use in laboratory animals; however, few tests in animals are well developed and have demonstrated ability to detect damage produced by hepatotoxic substances. Those tests that do detect liver dysfunction and damage are of particular interest in developing a screening program for hepatotoxicity and are discussed in detail in this report.

The measurements of liver damage have been grouped into three categories based on the structural, functional or biochemical changes that can be measured. The section on morphological damage indicators (Section 2.0) describes changes in liver morphology that indicate hepatotoxicity. These alterations in morphology may include zonal, diffuse or massive necrosis; steatosis; various forms of cirrhosis, and lesions of the venules.

The functional activities of the liver which can be assessed are excretion, storage and synthesis. The tests for excretion are discussed in Section 3.0 and those for storage and synthesis in Section 4.0. Tests for the excretory capacity of the liver involve the excretion of injected dyes or of endogenous bilirubin. Measurements of storage function include analyses for serum iron. Synthetic activities are evaluated by measuring changes in blood levels of such products as clotting factors and lipoproteins.

The biochemical tests discussed in Section 4.0 are presented in four subsections: serum enzymes, metabolism of normal and xenobiotic materials, metals in serum, and several in vitro procedures. Since the liver has a considerable functional reserve capacity, tests that are capable of measuring this reserve capacity will be described.

In Section 5.0, entitled Conclusions and Recommendations, criteria are defined for assessing the suitability of tests for inclusion in a hepatotoxicity screening program. Those tests which satisfy these criteria are selected and classified according to a second set of criteria as belonging in Levels I, II or III of a tiered testing program. The tiered testing scheme that is presented is based upon a critical, comparative analysis of all of the liver tests currently used in small laboratory animals.

Selected information concerning each test has been summarized in tabular form and is presented in appendices A through D. This information includes:

- the specific parameter measured
- the species in which the test has been performed, and
- the substances that have been tested for toxicity or used to elicit a toxic response.

The "comments" column of each table contains information distilled from the body of the text, that is pertinent to the suitability of the test for assessing liver toxicity.

The information contained in this report has been compiled from published and unpublished reports and communications with individuals active in the development or application of techniques for the determination of liver damage. A companion directory of some individuals and organizations involved in hepatotoxicity testing in animals has been compiled solely from personal communications, so that only current activities of organizations and researchers would be represented.

## 2.0 MORPHOLOGIC INDICATORS OF HEPATIC DAMAGE

Changes in the form and structure of the liver in man or animals often occur as a result of their exposure to toxic substances. The variety of chemicals producing morphologic changes in the liver is shown in Table 2-1. Observable changes in the morphology of the liver that may be indicative of toxic chemical effects will be described in this section. These changes will be discussed beginning with gross morphology, progressing through those seen with a light microscope to those detected by electron microscopy. The morphologic changes in the liver, reported in a collection of 95 toxicology papers, are given in Table 2-2, as presented by Gray (1976).

### 2.1 Gross Morphology

The liver is a dark, reddish-brown organ and is the largest gland in mammals. In man, it is a wedge-shaped structure of soft, friable tissue encased in a membrane. It is located on the right side of the body below the rib cage and extends to the middle of the left side. In adult humans, it normally weighs between 40 and 60 ounces, and is about nine inches by seven inches, but it may become several times larger from toxic chemical effects. In experimental animals, these dimensions are in the same proportions to total body weight as they are in man. In all species, the liver has five lobes that are more or less sharply delineated, depending on the species. In mammals the gall bladder, which serves as a reservoir for the bile, is located below and behind the liver, except in the rat, which has no gall bladder.

TABLE 2-1  
CHEMICALS SHOWN TO PRODUCE MORPHOLOGICAL  
CHANGES INDICATIVE OF LIVER INJURY

---

Aldrin	2'-β-Hydroxyethyl thioether analogue of griseofulvin
Androsterone	
Benzydamine	Halothane
Butylated hydroxyanisole	Hydrazine
Butylated hydroxytoluene	Isogriseofulvin
Chlordane	Meclizine
Chlorphenothane	Mephenytoin
Chlorpromazine	Penthrane (methoxyfluran)
Coramine	Phenobarbital
Cortisone	Phenylbutazone
Dimethylnitrosamine	Polycyclic aromatic hydrocarbons
Ditertiarybutylmethylphenol	SKF 525-A
Estradiol	Testosterone
Ethanol	Thiopental
Ethyl chlorophenoxyisobutyrate	Thiourea
Griseofulvin	Thyroxine

---

Source: Barka and Popper (1967) and Gray (1976).

TABLE 2-2  
TYPES OF LIVER INJURY REPORTED IN THE LITERATURE  
(1959-1974)\*

TYPE	NO. OF REPORTS
Necrosis	
Focal	9
Centrilobular	25
Midzonal	2
Peripheral necrosis	6
Submassive	3
Massive	1
Fatty change	7
Cholestasis	2
Cholangiotoxic	14
Progressive	
Cirrhosis	1
Neoplasia	12
Hemosiderosis	2
Kupffer cell deposition	2
Porphyria	3
Enlarged common bile duct	2
Granuloma	1
Degenerative hepatocytes	4
Diffuse cloudy swelling	1
Perinuclear vacuolization	1
Total in 95 papers	98

\* In Toxicology and Applied Pharmacology, 1959-1974.

SOURCE: Gray, 1976

Two vessels feed into the liver--the hepatic artery, which provides oxygenated blood; and the portal vein, which brings in nutrients. The blood exits by way of a capillary network ending in the hepatic vein. Lymphatic sinusoid vessels permeate the entire structure. The hepatic duct, which collects the secreted bile from the canalliculi, is connected to the gall bladder except in the rat, in which it empties directly into the intestine.

A change in the morphology of the liver depends upon the nature of the toxicant, the dose, the time of autopsy after the dose, the age and sex of the animal and several other factors. Often gross examination of the liver following exposure to an hepatotoxin will reveal an enlarged liver (hepatomegaly), which may or may not be discolored. Hepatomegaly may be due to enlargement of the liver cells (hypertrophy) or to a proliferation of cells (hyperplasia). These causes can be distinguished by differential staining or quantitative separation of the DNA, which increases per unit volume of tissue in hyperplasia and decreases with hypertrophy (Barka and Popper 1967).

Hypertrophy of the liver is enlargement due to an increase in the size of its constituent cells. Liver cells may increase in size because of a deposition of materials such as fat droplets, an increase in structural elements, or an increase in the levels of fluid within the cells. Hypertrophy is normally a dose-dependent response which reverses when administration of the chemical is discontinued, unless the injury has caused a permanent change, as in

fibrosis or necrosis. However, some substances such as iproniazid and penicillin may cause idiosyncratic reactions which are not dose related. In general, those chemicals which are not metabolized or are easily metabolized produce less enlargement than those metabolized more slowly and which are lipid soluble. Examples of the latter include ethanol, halothane and polycyclic hydrocarbons (Barka and Popper 1967).

Hyperplasia, an increase in the number of liver cells, is associated with the stimulation of DNA synthesis following the exposure of the liver to substances such as isoproterenol. It has been suggested (Barka and Popper 1967) that the reduction in the function of existing hepatocytes may stimulate cell division and production of new hepatocytes.

Liver enlargement may also result from other conditions such as the collection of fluid between cells (e.g., cholestasis) and from tumors or abscesses.

The liver may be discolored to a yellowish or tawny brown by the retention of bile (cholestasis) a response elicited by organic arsenicals. Retention of fats (steatosis), as caused by ethanol or mycotoxins, may also change the color. Fibrotic, cirrhotic or other areas with limited blood supply will appear pale or orange colored. These changes can arise, for example, from tannic acid or carbon tetrachloride (Plaa 1975a). Glycogen accumulation may appear as clear areas (Dobbins 1972).

## 2.2 Light Microscopy

Light microscopy is, at best, a semiquantitative art based on professional judgements of gradations of changes in the morphology of tissues; however, it is an essential and valuable component of toxicity studies. Seen under a microscope, the liver consists of roughly hexagonal lobules, each having in the center a branch of the portal vein and three vessels at each corner consisting of a branch of the portal vein, a hepatic arteriole and a bile duct. Material flow is outward from the central portal vein through the individual hepatocytes to the peripheral vessels (Plaa 1975; Robbins 1974). Chemical effects on any of the intervening membranes in these pathways can alter the function and/or structure of any or all of the downstream components.

Liver injury from short-term exposures to chemicals may produce either or both of two general kinds of morphologic changes: (1) accumulation of excretory or secretory products, and (2) damage to the cells or their components. As an example of type 1, ethionine produces steatosis (fatty liver) by interfering with the excretory mechanism. The fat droplets may accumulate in a single lobe or zone or may be widespread. Cholesterasis--the accumulation of bile pigment--is a frequent effect of steriods, which may be accompanied by aggregates of inflammatory cells in the portal area (Plaa 1975a; Zimmerman 1976). Illustrative of type 2 cell damage is necrosis in the central lobe (the most common site of chemical injury) which can

be caused by substances such as thioacetamide or tannic acid. Many chemicals such as ethanol and carbon tetrachloride will lead to both effects (Gray 1976; Plaa 1975a; Zimmerman 1976). Some compounds are known to produce hepatomegaly without visible alterations by light microscopy. For these compounds, electron microscopy may reveal structural changes (See Section 2.3).

Infarcts--localized necrosis from interruption of the blood supply--are seldom seen in the liver of mammals, because there is sufficient oxygen in either the portal vein or the hepatic artery to maintain the tissue (Robbins 1974).

Morphologic changes from short-term tests may be precursory, and predictive of effects from long-term exposures. Generally, the morphologic changes from short-term exposures are degenerative, whereas long-term exposures generally involve proliferative responses, especially increases in the number and form of hepatocytes, and may include neoplasia (Plaa 1975).

### 2.3 Electron Microscopy

The application of electron microscopy to pathology in the early 1960s provided not only a more detailed view of structures than light microscopy, but also an opportunity to quantify ultrastructural changes. The electron microscope now permits the correlation of biochemical and morphologic alterations, leading to a better understanding of mechanisms of toxicity (Gray 1976; Weibel et al. 1969).

Electron microscopy of hepatocytes reveals a network of channels bound by membranes extending from the nucleus to the external membrane of the cell. This network constitutes the endoplasmic reticulum, which appears as two types--smooth and rough--depending on the number of ribosomes attached to the surface. Ribosomes are bundles of ribonucleoprotein involved in protein synthesis (White, Handler and Smith 1968). In a study of ethanol ingestion by rats, Oudea, et al. (1973) saw no changes under light microscopy; however, electron microscopic morphometry indicated a 25% increase in smooth endoplasmic reticulum and a 34% decrease in the rough endoplasmic reticulum.

The largest organelles shown by the electron microscope are the mitochondria. They usually appear as dense rods about  $0.5 \times 3$ , but may also be seen as filaments or spheres. They are the site of respiratory and oxidative enzyme activity involved in energy transfer. Mitochondrial response to toxic chemicals, such as ethanol, is usually enlargement rather than proliferation. Cytoplasmic poisons, like heavy doses of carbon tetrachloride, cause dissolution of the mitochondria.

Hydrolytic enzymes are associated with the lysosomes, which are smaller bodies than the mitochondria. Damage to the lysosomes generally occurs late in the process of necrosis and results in release of the enzymes, indicating that they may not be involved in the necrotic process of the liver tissue (Plaa 1975a).

Details of an irregular structure of fibrils and vesicles in the hepatic parenchymal cell, known as the "Golgi apparatus" or "Golgi complex", are also visible under the electron microscope. Robbins (1974) describes electron microscopy studies of this structure, which abuts the cisternae of the endoplasmic reticulum, and which is involved in the secretory functions of the liver. These studies have shown the Golgi apparatus receiving granular and amorphous proteins from the rough endoplasmic reticulum, separating them, and directing their movement out of the cell. The granular proteins, which appear to be plasma proteins, are secreted into the space of Disse, from which they move into the vascular sinusoids. Microvilli from the hepatic artery and the portal vein structures protrude into the space of Disse. The amorphous proteins are transported by the Golgi complex from the rough endoplasmic reticulum to the lysosomes. This suggests that the amorphous proteins are enzymes. Observation of changes in these mechanisms can be useful in understanding the toxic action of chemicals. These movements are consistent with the studies of ethanol hepatotoxicity by Dobbins (1972) and Oudea (1973). Plaa (1975a) discusses how electron microscopy has shown that the cisternae of the rough endoplasmic reticulum are dilated by carbon tetrachloride and that the microsomal lipid structures are also altered. Concomitant biochemical studies showed a decrease in enzymic activity that correlated with the structural changes.

The early stages of cholestasis can rarely be identified in rodents by light microscopy. Bile pigment deposition following oral doses of ANIT ( $\alpha$ -naphthylisocyanate) is readily seen by the electron microscope along with changes in membranes of the hepatocytes (Plaa, 1975a).

Plaa (1975a) in his discussion of hepatotoxicity, gives examples of chemicals affecting the structure and/or function of these various organelles within the hepatocyte. These examples are shown in Table 2-3.

Gray (1976) summarizes the descriptive advantages of electron microscopy of liver specimens in safety evaluations, particularly with serial needle biopsies. These include the ability to provide (1) more precise and detailed pathologic descriptions; (2) observation of rapid, transitory, or sequential changes; (3) clues to biochemical or pharmacological mechanisms; (4) monitoring of the metabolic state and (5) definition of the limits of tolerance.

Serial biopsies, as proposed by Gray, provide an opportunity to follow the progress of liver injury from chemicals in the same animals. This procedure is particularly useful in larger animals, such as the dog, since serial sacrifices need not be done. These data can be correlated with biochemical measurements.

Techniques for the quantitative characterization of the morphology of rat livers were described by Weibel et al. (1969) and Loud (1968). Normal values for some of the organelles in rat liver are

TABLE 2-3  
EXAMPLES OF HEPATOTOXINS AFFECTING VARIOUS  
ORGANELLES

ORGANELLES AFFECTED	COMPOUND
Endoplasmic reticulum	Carbon tetrachloride Thioacetamide Dimethylnitrosamine Phosphorus Ethionine Dimethylaminoazobenzene Allyl formate Pyrrolizidine alkaloids
Mitochondria	Carbon tetrachloride Pyrrolizidine alkaloids Ethionine Allyl formate Tannic acid Phosphorus Hydrazine Dimethylnitrosamine
Lysosomes	Carbon tetrachloride Pyrrolizidine alkaloids Beryllium
Nucleus	Pyrrolizidine alkaloids Dimethylnitrosamine Hydrazine Beryllium Aflatoxin

Adapted from: Plaa 1975a

shown in Table 2.4. These morphometric data provide a standard for comparing similar measurements of liver tissues from animals exposed to chemicals.

Quantitative morphometry has been correlated with biochemical data, for example, in studies of the effect of ethanol ingestion on rat liver by Dobbins et al. (1972). The use of light microscopy failed to distinguish between the livers of control and ethanol-treated animals. The electron photomicrographs, however, revealed increases in the volume of mitochondria, lysosomes, peroxisomes, and lipid droplets, and a decrease in the volume of glycogen. Measurements of the surface area of these organelles paralleled the volume data. Biochemical analyses indicated a two-fold increase in lipids and in aniline hydroxylase. Our knowledge of the location of specific enzymes within these organelles is not yet complete enough to permit a detailed explanation of the correlations between morphologic and biochemical changes.

Increased surface area of the smooth endoplasmic reticulum at the expense of the rough endoplasmic surface area was first observed with phenobarbital. The correlation of this shift with the induction of enzymes for processing exogenous chemicals strongly indicates that the smooth reticulum is the site of these induced enzymes. Thus, a change in the relative areas of these surfaces is a measure of the animal's response to chemical exposure (Barka and Popper 1967).

TABLE 2-4  
NORMAL QUANTITATIVE VALUES FOR SELECTED  
ORGANELLES IN THE RAT LIVER

<u>Component</u>	<u>Mean/ml Tissue</u>	<u>Mean/100 gm body wt.</u>
Hepatocyte volume	0.831 $\text{cm}^3/\text{cm}^3$	2.79 $\text{cm}^3$
Hepatocyte surface	0.284 $\text{m}^2/\text{cm}^3$	0.96 $\text{m}^3$
Nuclear volume	0.050 $\text{cm}^3/\text{cm}^3$	0.17 $\text{cm}^3$
Rough ER volume	0.0785 $\text{cm}^3/\text{cm}^3$	0.264 $\text{cm}^3$
Rough ER surface	6.25 $\text{m}^2/\text{cm}^3$	20.93 $\text{m}^2$
Smooth ER volume	0.049 $\text{cm}^3/\text{cm}^3$	0.163 $\text{cm}^3$
Smooth ER surface	4.65 $\text{m}^2/\text{cm}^3$	15.61 $\text{m}^2$
Mitochondria volume	0.0116 $\text{cm}^3/\text{cm}^3$	0.38 $\text{cm}^3$

Adapted from: Weibel et al. 1969

#### 2.4 Species Variations in Morphology

Although mammalian toxicology is only concerned with species in a single phylum, there are wide variations of morphology, physiology, and biochemistry among the numerous mammalian species.

The majority of hepatotoxicity tests have been done using the rat and much has been learned about hepatotoxic response in this model; however, the rat has been used principally because of convenience and economy, not necessarily because it is the best animal model for predicting human response to toxic chemical substances. In hepatotoxicity testing, structural and ultrastructural changes in the liver produced by toxic agents have been investigated in mice, rats, hamsters, guinea pigs, rabbits, dogs, cats, cattle, swine, horses, sheep, and birds.

As noted earlier a major morphologic difference between the rat and man is that the rat has no gall bladder. Also, the rat liver is generally not as readily affected by chemicals as is that of the dog, or that of man. Gray (1976) states that the rat is preferable for detecting enlargement of the liver, and this effect generally provides a more sensitive test for detecting hepatic damage in this animal model than either serum enzyme activities or light microscopic changes. Gray further reports that the dog, especially the beagle, is more sensitive and reliable for predicting effects in man, and that the monkey is more variable and less susceptible than the dog with regard to liver injury by chemicals.

## 2.5 Summary

Morphologic examination is an essential aspect of the assessment of toxic substances in laboratory animals. It provides at least a tentative and often a definitive description of the toxic effects of chemical substances and may provide information on the mechanism of toxic action. Nonetheless, the extent to which morphologic examination is implemented is a matter of judgement, in which the purpose of the study as well as the associated increases in manpower and finances must be considered.

The liver is the principal detoxification organ of the body and is the organ most commonly affected by chemicals. These effects can be observed with increasing sensitivity, understanding, and expense as the degree of magnification increases from that of the naked eye, to the light microscope, and to the electron microscope, which has magnification power of nearly 450,000. Gross morphology alone is generally considered sufficient for exploratory studies and acute toxicity testing. A pathologist or an appropriately trained individual should assess the gross changes. Light microscopy may also be necessary in acute toxicity testing to provide a more definitive description of liver lesions. Light microscopy is the minimum required for sub-chronic and chronic investigations. At present, electron microscopy is usually reserved for research into mechanisms of action because of the costs and time involved.

Rats are the most commonly used animal for acute and chronic studies of systemic toxicity. Dogs, especially pure bred beagles, are being required increasingly for regulatory studies of chronic toxicity as well as for research. Dogs are more sensitive to the effects of hepatotoxins than rats; however, they are not as convenient as the rat and are much more costly to use. The rat is the recommended species for short-term testing. Other species (e.g., primates, guinea pigs, rabbits, and hamsters) are rarely used in short-term hepatotoxicity testing.

### 3.0 FUNCTIONAL INDICATORS OF HEPATIC DAMAGE

The three basic functions of the liver are (1) secretion of bile into the gastrointestinal tract; (2) filtration of the blood, and storage of such vital components as glycogen, vitamins and iron; and (3) biochemical reactions related to the majority of metabolic systems of the body. A number of tests have been used to detect and monitor hepatic functional damage in experimental animals. Only a few testing techniques have been sufficiently sensitive, specific, reliable, and simple to perform to be used regularly in evaluating hepatotoxicity in animals. Most functional testing investigates the rate of hepatic elimination of either exogenous (e.g., sulfobromophthalein, indocyanine green) or endogenous (e.g., bilirubin) substances. The following sections describe the tests used to monitor hepatic function.

#### 3.1 Dye Clearance Tests

Measurements of uptake from the blood and excretion of exogenous dyes through the biliary system have been used to monitor liver function. The most widely used dye for this purpose in the past has been sulfobromophthalein (BSP); however, more recently, indocyanine green (ICG) has been used.

##### 3.1.1 Sulfobromophthalein Excretion

BSP is administered intravenously and the concentrations in the blood at a single or at multiple samplings are determined spectrophotometrically at 580 nm. In most screening protocols, the amount

of dye remaining in the plasma is determined at a set interval after injection. Several determinations are made when the kinetics of dye elimination are being examined. A difficulty in animal testing is taking accurately timed blood samples, because BSP is rapidly eliminated in laboratory animals such as the rat and rabbit. In obtaining blood samples, even a difference of only 20 seconds can cause abnormal readings (Street 1970). BSP levels are expressed as the percent of dose retained in blood. In most laboratory animals, when an appropriate dose is administered, retention of over 5 percent of the dose in 45 minutes would be abnormal.

As would be expected, there are considerable species variations in the ability of different species of laboratory animals to remove BSP from plasma. Both the rat and rabbit clear the material quickly, while the dog clears it relatively slowly. The rat and rabbit excrete BSP at approximately 1 mg/min/kg, while the dog excretes this substance at approximately 0.1 mg/min/kg. For this reason, the optimum dose of BSP will vary, depending upon the species and strain of animal used. An incorrect selection of a dose can produce misleading results (Plaa 1968). One reason for this difference between animal species is that in some animal species, substantial amounts of BSP may be excreted by the kidneys in addition to active biliary excretion by the liver, thus influencing the rate of clearance from plasma. The rate of BSP excretion can be affected by a number of factors in addition to the dose of the test substance. BSP is

conjugated in the liver with glutathione, and therefore its rate of excretion is dependent upon the availability of glutathione for conjugation. The administration of or exposure to drugs, chemicals and enzyme inducers, can either increase or decrease dye excretion rates. Other factors which may affect excretion rates are: hepatic blood flow changes, extrahepatic disease, cardiac failure, hepatomegaly, fever and shock.

Even though a number of factors influence BSP retention, it is a sensitive and useful technique for assessing liver function and it should have application in a screening program. Table B-1 in Appendix B lists a number of substances that have been examined using BSP clearance techniques.

### 3.1.2 Sulfobromophthalein Transport Maximum

The transport maximum ( $T_m$ ) for BSP has been used to a limited extent in dogs (Wheeler et al. 1960), and in rabbits and rats (Klaassen and Plaa 1969), for the evaluation of liver function. The  $T_m$  is a serum concentration at which the elimination of BSP attains a constant maximal rate. This requires BSP infusion at increasing rates until the transport mechanisms are saturated. The  $T_m$  is then determined. This technique is too involved and not sufficiently sensitive to be practical in a routine testing program.

### 3.1.3 Indocyanine Green Excretion

A dye that has more recently come into use is indocyanine green (ICG). ICG is rapidly excreted in bile in an unconjugated form at a

rate similar to that of BSP. ICG is not removed from the body by extrahepatic means, as is BSP. It is totally excreted into the bile. For these reasons, ICG is now employed in some laboratory animal studies in place of BSP (see Table B-1.1 in Appendix B). As with BSP, ICG is administered intravenously and its disappearance from the blood is measured spectrophotometrically in blood samples taken at accurately timed intervals. ICG has the disadvantage of being unstable in aqueous solutions. Its decomposition can be prevented by mixing it with serum or an albumin solution, since it is rapidly and completely bound to albumin and other serum proteins.

#### 3.1.4 Indocyanine Green Transport Maximum

The transport maximum ( $T_m$  for ICG) has been used to a limited extent in laboratory animals (Hargreaves 1966). As with BSP, the plasma level of ICG can be sufficiently increased to saturate the biliary transport mechanisms and then the  $T_m$  can be determined. The measurement of  $T_m$  for ICG is too involved and not sufficiently sensitive to be useful in the routine screening of chemical substances for hepatotoxic activity.

#### 3.1.5 Rose Bengal Excretion

A third dye, rose bengal, is historically interesting--it was the first dye to be used in liver function testing. The dye is administered intravenously, and its elimination is measured in animals either through blood clearance procedures or through detection in the contents of the intestine. BSP or ICG has replaced rose bengal for

most studies. A new method which uses labeled ( $^{131}\text{I}$ ) rose bengal is now available. The rate of accumulation and/or clearance is recorded by use of a scintillation counter. Rose bengal  $^{131}\text{I}$  is useful in specialized research studies and is a relatively expensive procedure for monitoring liver function.

### 3.2 Bilirubin Clearance

Bilirubin is present in the serum and originates from the breakdown of hemoglobin in red blood cells, a process that takes place in some of the cells of the reticuloendothelial system. The plasma albumin, to which the bilirubin becomes attached, transports it to the liver. Bilirubin is conjugated in the liver with glucuronic acid, whereupon the hepatocytes actively excrete it into the bile canaliculi. Some unconjugated bilirubin is also excreted into bile by the hepatocytes. Total serum bilirubin levels include both conjugated and unconjugated bilirubin. The present-day procedure is to determine unconjugated and total bilirubin, and assume that the remainder is conjugated.

Levels of serum bilirubin are quite similar in dogs and humans (normal 1.0 mg/dl), but all other common laboratory mammals have very low levels, including rats (Street 1970); mice (Casals and Olitsky 1946); and monkeys (Benjamin and McKelvay 1978). These low normal levels make small increases difficult to determine. In laboratory animals, however, it is useful to determine if urobilinogen (formed

from bilirubin in the intestine by bacterial action) is present in feces, and to use that measurement to aid in the diagnosis of liver damage.

Urobilinogen in the urine is also a useful indicator of liver damage. Urobilinogen, formed in the intestine and reabsorbed into the plasma, is normally eliminated via the liver. When hepatocytes are stressed or damaged, urobilinogen accumulates in the plasma and is excreted by the kidneys. However, increased hemoglobin breakdown in cases of increased red blood cell destruction may also lead to urobilinogen in the urine.

Some substances, when present in the body, can interfere with (a) hepatocellular uptake of bilirubin (e.g., flavaspidic acid); (b) its excretion (e.g., anabolic steroids); or (c) both its uptake and excretion (e.g., rifampicin) (Davidson et al. 1979). Thus, chemicals can, by various means, cause rises in serum, urine, and fecal forms of bilirubin.

All the methods used for bilirubin assay depend upon formation of azobilirubin using diazotized sulfanilic acid. The intensity of the purple color (azobilirubin) formed in this method is determined by colorimetry or spectrophotometry, and is proportional to the bilirubin in the sample. The most popular methods of estimating the serum (plasma) bilirubin are the Jendrassik-Cleghorn and Ducci-Watson

modifications of the 1937 Malloy-Evelyn techniques. Davidson et al. (1979) have reviewed and described these procedures. Studies where bilirubin metabolism and clearance have been examined are shown in Table B-1.2 in Appendix B.

Urobilinogen in the urine is also detected using methods involving the diazo reaction. Rather crude, but very useful methods, which are popular in the animal laboratory, are the "dip sticks" impregnated with diazo reagent. If urine is positive, it is quite likely to contain urobilinogen, which is abnormal. In exposure to hepatotoxins, including certain pharmaceuticals, a positive test for bilirubinuria may be the earliest indication of liver damage (Bradley et al. 1979).

### 3.3 Biliary Transport Maximum

The bilirubin transport maximum ( $T_m$ ) can be determined by infusing sufficient bilirubin to saturate the transport and conjugation mechanisms of the liver. In this technique, the animal is anesthetized, and the femoral vein and bile duct are cannulated. The bilirubin is infused into the femoral vein and bile samples are collected from the bile duct. The maximum amount of bilirubin excreted in the bile per unit time as the infusion level is increased is the transport maximum ( $T_m$ ). The use of  $^{14}C$ -bilirubin simplifies the analysis of samples. This test is a sensitive measure of hepatic function (Zimmerman 1979a), but it is laborious and complex to perform and is

currently used only for research purposes. However, it may have application in more advanced levels of screening (Level II or Level III) of a testing program.

#### 3.4 In Vitro Techniques

A number of in vitro models are available for use in the study of hepatic function. These include perfused liver, liver slices, suspended or cultured isolated fresh hepatocytes, liver homogenates, and isolated organelles from hepatocytes. Each of these model systems is described in detail in Section 4.4 and studies using these model systems are shown in Appendix D. Most studies using these model systems have dealt with liver metabolic activity. Nevertheless, the in vivo techniques described above for dye clearance, bilirubin clearance, and bile flow can be adapted to some of the in vitro models.

#### 3.5 Radioisotopic Techniques

Radioisotopes have been used extensively in studies of hepatic physiology and function. Radioisotopes have been especially useful in monitoring blood flow to the liver, and in determining the configuration and size of the liver by using either scintillation or imaging techniques. Many of the exogenous substances used in the various tests described in this document have been labeled with radioactive isotopes. Most common among these substances are  $^{14}\text{C}$ -bilirubin,  $^{14}\text{C}$ -cholylglycine,  $^{58}\text{Co}$ - or  $^{60}\text{Co}$ -cyanocobalamin,  $^{131}\text{I}$ -BSP and  $^{131}\text{I}$ -rose bengal. The use of radiolabeled

substances simplifies the analytical techniques necessary to measure the rates at which these substances disappear from the blood, accumulate in the liver, or are eliminated and excreted from the body.

Radioactive colloids, which are taken up by reticuloendothelial cells, are used for nuclear imaging. Some of the radiolabeled sulfur colloids which have been used include gold (<sup>198</sup> Au or <sup>199</sup> Au), indium (<sup>113m</sup>In) and technetium (<sup>99m</sup>Tc). The areas in the liver that fail to accumulate radioactivity at the time of nuclear imaging represent pathological processes.

<sup>131</sup>I and <sup>99m</sup>Tc-labeled albumin and <sup>133</sup>Xe remain in the blood, and have been used for liver perfusion studies to measure blood flow rates (Zimmerman 1979a).

The investigation of liver dysfunction using radioactive substances, except where noted in other sections, has been used principally for research purposes. These techniques would not be useful currently for routine screening, except possibly in the later stages of a screening program where hemodynamic measurements are necessary in studying the mechanisms of damage.

### 3.6 Summary

Many different tests have been developed to monitor hepatic function. Only a few tests, however, have been sufficiently sensitive, specific, reliable, and simple to perform to be used in routine hepatic testing. The tests described in this section investigate the

rate of hepatic elimination of exogenous dyes (e.g., sulfobromophthalein, indocyanine green) and endogenous substances (e.g., bilirubin).

The most widely used dye for monitoring liver function in the past has been sulfobromophthalein (BSP). BSP is conjugated in the liver with glutathione before elimination; thus its elimination is dependent upon the availability of glutathione in the liver. When glutathione is available for conjugation, BSP is rapidly eliminated from the plasma. Even though a number of factors influence BSP elimination rates, it is a sensitive measure of liver function.

In recent years, BSP in animal laboratory work has been partially replaced by indocyanine green (ICG). ICG is also rapidly eliminated from the plasma; however, it is eliminated in an unconjugated form. Therefore, its excretion is not dependent upon the availability of a conjugation mechanism. ICG is also not affected by as many other factors as BSP. Transport maxima ( $T_m$ ) (i.e., the maximum amount of dye excreted per unit time) have been measured for both BSP and ICG by infusing sufficient dye to saturate the biliary elimination mechanisms. The  $T_m$  determinations have not been widely used and are not considered useful for routine toxicity screening.

Rose bengal was the first dye used in liver function testing. It was not considered as sensitive as BSP, so it was replaced and not used much for many years. Recently,  $^{131}I$ -labeled rose bengal has been used to a limited extent for specialized studies. The other dyes (i.e., BSP and ICG) can also be radiolabeled to simplify the analytical techniques.

Bilirubin is an endogenous substance present in the serum and originates from the breakdown of hemoglobin in red blood cells. It is conjugated in the liver with glucuronic acid and eliminated in the bile. Most common laboratory animals such as rats, mice, and monkeys have very low normal levels of bilirubin in serum. Accordingly, small increases due to hepatic damage are difficult to detect in these animals. A more useful and sensitive technique in laboratory animals is the detection of urobilinogen (formed from bilirubin) in urine. Urobilinogen in urine is abnormal and is one of the earliest indications of liver damage. Simple "dipsticks" are available for detecting urobilinogen in urine.

The bilirubin transport maximum ( $T_m$ ) can be determined by infusing sufficient bilirubin to saturate the biliary transport system. This technique is laborious to perform and is currently used only for research purposes. Nevertheless, it is a sensitive measure of hepatic function and may be performed using  $^{14}\text{C}$ -labeled bilirubin, which simplifies the analysis of samples.

All of the techniques described in this section can be adapted for use in in vitro model systems, such as perfused liver and liver slices. These in vitro models provide the advantage of carefully controlled biochemical parameters; nevertheless, they are only used for research purposes and would not be considered useful for routine hepatic screening of toxic substances.

#### 4.0 BIOCHEMICAL INDICATORS OF HEPATIC DAMAGE

The liver is an organ of diverse biochemical activity. It is the principle detoxification organ of the body and performs many metabolic functions related to carbohydrate, lipid and protein metabolism and the storage of metabolic products. Tests that monitor these biochemical metabolic mechanisms can provide information concerning hepatic dysfunction and damage.

Sections 4.1 and 4.2, respectively, describe the use of serum enzymes in detecting hepatic damage and the monitoring of carbohydrate, lipid, protein and xenobiotic metabolism for hepatic dysfunction. Section 4.3 describes methods for the monitoring of serum metal levels as measures of hepatotoxicity. The final section (Section 4.4) describes the use of in vitro model systems in the biochemical assessment of hepatotoxic potential.

##### 4.1 Serum Enzymes

Enzymes are proteinaceous catalysts which are essential to most of the chemical reactions in living organisms. They are normally present in tissue cells and body fluids and are at low levels in blood plasma. These low levels of circulating plasma enzymes are most probably not biologically significant. They represent those enzymes released during natural cell attrition. However, when an unusually large number of cells are destroyed or injured, a relatively large quantity of one, or several enzymes may be released into tissue fluid and plasma. When cells are injured, Serum Cholinesterase

formation is impaired. These increases, or decreases, in plasma enzyme levels form the basis of enzyme tests to detect cellular malfunction or injury.

The effective use of enzyme levels for diagnosis of liver cell dysfunction in human and animal disease arose with the work of Karmen et al. (1955) who published descriptions of methods for determining blood transaminases, and associated their fluctuations in plasma with myocardial infarction (humans) and liver disease (humans and animals). That began an intense search for serum and tissue enzyme changes which could be associated with organ (especially liver) dysfunction.

The exact mechanisms by which enzymes are released is not fully understood. The stressed cell may release enzymes because of toxic effects on its functions (such as increased cell membrane permeability), internal biochemical changes, or cellular degeneration (necrosis) (Cornish 1971)).

The Commission on Enzymes of the International Union of Biochemistry has defined an International Unit (U), sometimes IU, as the amount of enzyme that catalyzes the conversion of 1 micromole (micro-equivalent) of substrate or coenzyme per minute under the defined conditions (temperature with optimal pH and substrate concentration) of the tests. Since enzymes are present in serum or tissue in very small amounts, methods of direct measurement are not readily available. Enzyme levels are therefore expressed in "activity units",

indicating their capacity to function as catalysts. They are determined in one of three ways: (1) increase in concentration of a product, (2) decrease in concentration of a substrate, and (3) rate of change in concentration of a coenzyme, which is a measure of the rate of reaction (Zimmerman 1979b). The third method is most commonly used because coenzymes are readily detected by U.V. spectrophotometry.

The great variety of methods, apparatus, instruments of measurement and differences in reported 'normal' or 'control' levels attest to the fact that determination of enzymes is still difficult, and at times controversial. Expert guidance and experience with laboratory procedures is essential to produce dependable results. Each laboratory usually sets up its own standards, quality control, and 'normal' values. In most instances, especially in animal laboratories, the exact normal level is not as important as is the detection of changes in activities.

In addition to genetic variations between species, sub-species and strains or breeds of experimental animals, a variety of physiological factors cause changes in enzyme activities. These variations may be minimized in part by using control groups--as with rats and mice--or by using each animal as its own control--as with dogs and monkeys--by determining enzyme activity before, and several times during the testing procedure. Not only do endocrine fluctuations, age, and sex alter enzyme activity, but animals may vary diurnally, seasonally, in different nutritional and disease states, and also

under the influence of many chemical and physical conditions. Simple variations in experimental procedures can alter results, such as the following: (1) difficulties in obtaining blood samples causing hemolysis of cells; (2) changes in sample pH; (3) samples standing at room temperature; (4) the presence of anticoagulants; (5) duration of storage, even at low temperatures; (6) presence of natural pigments in the plasma; and (7) presence of lipids or any unusual plasma contents.

The amount of an enzyme in circulating plasma at a given time depends upon several factors, including (1) size of the organ producing it; (2) the proportion of that organ which is being induced to release it; (3) the time of measurement (some cells release measurable amounts within minutes, some take hours or days); (4) whether there are enzyme inhibiting or destructive factors present; (5) whether the injury causes increased or decreased production, or increased or decreased release; (6) whether one or more organs is injured; (7) whether cell permeability is altered; (8) the extent to which excretion into bile, intestine, or kidney is increased; and (9) viral interference with uptake of the enzyme, (e.g., lactic dehydrogenase by the reticuloendothelial system).

Enzyme measurements have the following advantages when compared with other tests for hepatotoxicity: less labor is expended; early damage may be detected; and serial measurements and injury comparisons can be made as well as measures of potentiation, adaptation, or

depression by other agents. Some of their limitations are: inability to differentiate between increased permeability of membranes and cell necrosis; uncertainty regarding the source of enzymes detected, since they may come from organs other than the liver; and failure to detect damage, since in some cases, injury may not cause any rise in enzyme levels. In laboratory animal testing, other means such as light- or electron microscopy, should be used in conjunction with biochemistry to determine which toxic lesions have occurred. The following sections will describe the most useful enzymes for detecting liver dysfunction and damage in laboratory animals.

#### 4.1.1 Transaminases

Hepatocellular malfunction can be detected by changes in the activity of serum glutamic-oxalacetic transaminase (SGOT)\* and serum glutamic-pyruvic transaminase (SGPT),\* which are found in blood plasma. Their activities are easily measured by a variety of instrumental techniques that detect the concentration of the coenzyme nicotinamide adenine dinucleotide (NADH), involved in the formation of oxaloacetate or pyruvate. Analytical methods include simple colorimetry (Reitman and Frankel 1957) and ultraviolet spectrophotometry (Wroblewski and LaDue 1956). Commercial autoanalyzers using micromethods are also available.

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\*The currently accepted nomenclature for glutamic-oxalacetic and glutamic-pyruvic transaminase (GOT and GPT) is aspartate and alanine transaminase or aminotransferase. GOT and GPT will be used throughout this document because they are widely used and understood.

The results of these enzyme determinations were previously expressed in Karmen Units; however, in the last few years International Units (IU) have become more commonly used. A Karmen Unit (KU) is a measure of rate of change in the concentration of NADH. These units may be converted to International Units or micromoles of NADH oxidized per milliliter of serum per minute (Karmen 1955).

SGOT is increased by cell damage in many tissues other than those of the liver, including brain, erythrocytes, kidney, skin, pancreas, cardiac and skeletal muscle tissues. The normal ranges for several species are shown in Table 4-1.

SGPT arises almost exclusively from liver cell damage; although myocardial damage may cause small increases. It is also quite consistent among species, as shown in Table 4-1. When the hepatocytes are damaged, the concentration of SGPT rises somewhat higher than does that of SGOT and it remains high longer.

Determinations of SGOT and SGPT are routinely used in clinical and experimental work even though some of the enzymes to be discussed below are more sensitive, reliable and specific to the liver. SGOT and SGPT values may be correlated in hepatotoxicity screening and are conveniently measured at the same time enhancing their significance. These are retained in the toxicology armamentarium because of familiarity, experience with deviations, years of accumulated records of control and experimental groups, availability of apparatus, and cost

TABLE 4-1  
MEAN LEVELS AND STANDARD DEVIATIONS,\*  
OF GOT AND GPT IN 22 ANIMAL SPECIES

Species	Enzyme	
	SGOT	SGPT
Man	9+3	7+3
Monkey (Range)	12 (10-13)	11 (4-18)
Dog (Range)	9+2	11+ 4
Ferret	46+9	14+ 3
Cat	13+2	12+ 8
Rat	52+18	7+ 2
Mouse (Range)	29+5	9+ 3
Guinea pig	23+8	13+ 2
Hamster	39+14	13+ 5
Rabbit	16+6	14+ 4
Pig (Range)	30 (25-35)	24 (13-36)
Cow	33+8	19+ 3
Sheep	36+21	8+ 3
Goat (Range)	21 (15-29)	(Single value)
Horse	62+21	5+ 1
Goose	13+5	5+ 2
Duck	21+16	6+ 2
Chicken	48+13	8
Pigeon	49+18	15+11
Snake (Range)	15 (10-25)	12 (4-20)
Alligator	56+11	6+ 2
Carp	52+20	8+ 4

Values expressed in International Units.

Excerpted from Zimmerman, Schwartz, Boley & West (1965).

\* Values derived from less than 5 animals are shown as the average with the range in parentheses.

of alternative procedures (Zimmerman 1978). Studies examining SGOT and SGPT levels in various animal species are shown in Table C-1 of Appendix C.

#### 4.1.2 Alkaline Phosphatase (ALP)

Alkaline phosphatase (ALP) is one of the many and varied phosphatases present in liver cells, which hydrolyze esters of phosphoric acid. ALP at pH 9.0 to 9.3 hydrolyzes monophosphoric esters, releasing inorganic phosphate. This phosphatase was the first enzyme to be associated with human disease. Gutman et al. (1936) associated it with osteoblastic (bone building) cellular activity. Since then, it has been found in many other tissues including liver, intestine, spleen, blood cells, kidney, and placenta. This enzyme's serum activity has been found to be higher in the young animal than in the adult. Levels vary inconsistently, probably reflecting variations in bone-building activity.

Alkaline phosphatase activity is determined by measuring the amount of phosphate ester hydrolyzed per unit time. The phosphate esters most commonly used in the analytical procedures include beta-glycerophosphate, p-nitrophenylphosphate, or phenolphthalein diphosphate. The amount of inorganic phosphate--or free chromogen (p-nitrophenol or phenolphthalein)--is then determined (Davidson et al. 1979). Determinations of ALP can be made easily on 0.25 ml of serum by spectrophotometry. Hemolysis of the sample does not interfere with the analysis. Normal adult rats have ALP activities in the

range of 200-850 IU (Street 1970). Normal activity in the dog is 20-85 IU (Benjamin and McKelvie 1978).

It was thought for some years that the liver acted only as the excretory organ for phosphatase eliminated from the bone and that liver disease interfered with that excretion (Butman 1959). It is now though that both functions--production and excretion--take place in the liver (Zimmerman 1978). Since the liver excretes ALP into the bile, any toxic injury to the liver cells involved in biliary excretion or congestive disorders of the biliary canaliculi will result in elevated plasma levels of ALP. Chlorpromazine damage in animals produces hepatocellular and hepatocanicular obstruction, leading to greatly increased levels of plasma ALP. Generalized necrosis from chlorpromazine, carbon tetrachloride, and pathological conditions such as hepatitis, are known to raise the ALP levels proportionally to the extent of damage (Korsrud et al. 1972). It can be expected that plasma ALP levels will rise in response to any disease or chemical insult that produces liver necrosis. ALP is thus a general indicator of liver function, which is especially sensitive to biliary obstruction. As would be expected, changes in ALP plasma levels correlate well with other measures of biliary excretion, such as the BSP and ICG clearance tests (Benjamine and McKelvie, 1978).

As noted above, ALP is found in many tissues and damage to them can also lead to increased plasma levels. For example, Keefe et al. (1978) found that orally administered chloroform (a vermifuge) acted

on the mucosa of the small intestine in dogs to release ALP into blood serum without causing microscopically detectable lesions. Thus, serum ALP activity may increase, due to nondetectable intestinal lesions in the absence of liver dysfunction. Keefe et al. (1978) stated that the dog is a good subject for ALP determinations, while the rat is not, since the levels for the rat are so much higher (3-10 times) than for the dog and small changes may go undetected. The dog has serum ALP activity similar to man and shows similar changes in ALP activity during hepatic dysfunction.

Since the ALP from the different tissues occurs as isoenzymes, it is possible to separate and identify them by various means: electrophoresis, selective absorption, solvent precipitation, denaturation, chemical affinity or inhibition techniques (Saini 1977). Such separations are used principally for research applications. They are too cumbersome and time-consuming for short-term toxicity testing. In spite of its non-specificity, ALP is commonly used because of its historical data base. Studies where ALP activity was examined are shown in Table C-1.1 in Appendix C.

#### 4.1.3 Ornithine Carbamyl Transferase (OCT)

Ornithine carbamyl transferase (OCT) was first described by Krebs and Henseleit in 1932 (Tegeris et al. 1969). This transferase was thought to be present in animal tissues only; however, in 1958, Reichard and Reichard described a method for its determination in

human serum. OCT is involved in nitrogen metabolism, specifically in the formation of urea, by catalyzing the reversible conversion of ornithine to citrulline.

OCT is a highly specific enzyme for the liver with only one percent of the total serum activity arising in the intestines and a few other tissues; nonetheless, it has not been as widely adopted to detect liver dysfunction and disease, principally because the analytical methods have not been adequately standardized and automated.

The methods for determining serum OCT activity involve monitoring the OCT-catalyzed breakdown of citrulline to ornithine in the presence of arsenate, liberating CO<sub>2</sub> (Reichard 1964). Current techniques use <sup>14</sup>C-labeled citrulline as the substrate, and the amount of <sup>14</sup>CO<sub>2</sub> released during reaction is estimated by scintillation counting (Reichard 1964; Korsrud et al. 1973; Drotman 1975).

Although normal serum levels are low, they rise spectacularly in liver cell necrosis. Tegeris et al. (1969) report that human toxic hepatitis patients have levels of 7 to 63 IU, compared with normal levels of 0-2.5 IU. In acute chemical liver necrosis in humans, the OCT rises steadily and persists for about three weeks (Wolf and Williams 1973).

In the dog, and swine, liver injury caused by carbon tetrachloride can raise the OCT activity 100 times the control level in 24 hours, and 500 times the control level in 48 hours (Benjamin and McKelvie 1978; Tegeris et al., 1969). The data show that in the dog

OCT levels parallel transaminase (i.e., GOT and GPT) levels, except that the percent rise is much greater with OCT. In 1975, Drotman published the results of a study on the effects of carbon tetrachloride on rat liver release of OCT. The OCT range of activity varied from 0.6-6.0 (control) to 620-3200 mole/24 hr/l serum when carbon tetrachloride was administered at a dose of 300  $\mu$ l/kg. DiVincenzo and Krasavage in 1974 determined OCT activity in normal dogs (0-39 IU); cats (0-4.7 IU); rats (0.2-1.6 IU); guinea pigs (0-8.9 IU); and rabbits (0.9-4.9 IU).

Considering the results of research using OCT as a measure of chemical liver injury in animals, it should be seriously considered as a highly specific liver microsomal injury test, although further development and standardization of the analytical techniques is needed before serum OCT can be monitored routinely in short-term screening programs. Studies examining OCT activity in various animal species are shown in Table C-1.2 of Appendix C.

#### 4.1.4 Iditol Dehydrogenase (ID)

ID catalyzes the reversible conversion of sorbitol to fructose, an important step in carbohydrate metabolism. Also known as sorbitol dehydrogenase (SDH), it is present in normal liver cells and in minimal amounts in serum, semen and skeletal muscle. ID appears in large quantities in serum when there is toxic, infectious, or hypoxic liver injury, making it a fairly specific indicator of liver cell damage (Wolf and Williams 1973). Asada and Galambos (1958) measured ID in

the liver cells and blood serum of rats that had been given carbon tetrachloride. The dose-related rise of the enzyme activity was rapid in serum (beginning in 24 hours), and the corresponding fall in tissue levels was also rapid when administration was terminated. The highest serum levels reached were about 500 IU in 48 hours.

Strubelt et al. (1978) determined serum ID activities in mice before and after treatment with ethanol and several other toxic chemicals. ID rose from 10 IU/l (control) to over 1100 IU/l after treatment with ethanol or bromobenzene. Korsrud et al. (1973) found that ID was the most sensitive serum parameter studied among OCT, ICD (isocitric dehydrogenase), GOT, GPT, MDH (malic dehydrogenase) and LDH (lactic dehydrogenase), when the chemicals thioacetamide, dimethyl nitrosamine, or diethanolamine were administered to rats. ID increased sooner after smaller doses of the toxic substances than any of the other enzymes mentioned above.

Methods for the detection of ID depend upon the rate of oxidation of the coenzyme NADH (nicotinamide adenine dinucleotide) to NAD. The rate of oxidation is estimated spectrophotometrically. Determinations can be made on 1.0 ml or less of unhemolyzed serum. ID activity in normal human serum is 1.0 IU/ml; in the normal mouse, it is 0.01 IU/ml and in the normal rat, it is 0.057 IU/ml (Strubelt et al. 1978); normal levels in other animals are not available. The differences among normal serum levels of this cytoplasmic enzyme may require adjustment of the substrate levels. Studies examining ID activity are shown in Table C-1.3 of Appendix C.

#### 4.1.5 Gamma-glutamyl Transpeptidase (GGT)

Gamma-glutamyl transpeptidase (GGT) transfers the gamma-glutamyl moiety from one peptide to another or to an amino acid. It is found in the hepatocyte and bile ductules of the liver, and in the pancreas, and kidney. Although the quantity is greatest in kidney tissue, the origin of serum GGT is the liver (Malherbe et al. 1977). The chief interest in this enzyme is that it originates mainly in the smooth endoplasmic reticulum (SER), rather than in the cytoplasm of the liver cell, and it responds to chemical substances which induce SER enzymes (Davidson et al. 1979). That property is of concern in laboratory animal testing since in some instances, it is necessary to use inducers to increase enzyme activity prior to or concurrent with administration of the test substance. Elevations in observed GGT levels may be attributable to the inducer and not the test substance. Methods of determination vary, but most of them require the use of a spectrophotometer. Normal ranges vary with each method and few control animal levels are available.

GGT is a sensitive indicator of hepatobiliary dysfunction and is a more sensitive and specific indicator of cholestasis than the transaminases, which respond to most kinds of liver damage (Davidson et al. 1979). It is not affected by bone diseases or other osseous changes; consequently, it is useful in evaluating the significance of elevated ALP activities.

Malherbe et al. (1977) studied the GOT and GGT activities in the serum of sheep with liver damage (lupinosis) produced by a mycotoxin. They found that GGT was most valuable in revealing early, low grade, acute and chronic intoxication while GOT gave a better indication of severe, acute damage and that GGT and GOT together gave the best information on the course of the liver toxicosis. Changes in activities of both enzymes also paralleled histopathologic changes. However, GGT is not considered by Davidson et al. (1979) to be useful in small laboratory animals such as rats, mice and hamsters because of low serum level concentrations even in the presence of liver damage. Nonetheless, it may be useful in experimental toxicity studies in laboratory animals (e.g., dogs) that show a large variability in serum ALP activities. Studies examining GGT activity in various animal species are shown in Table C-1.4 of Appendix C.

#### 4.1.6 Lactic Dehydrogenase (LDH)

Lactic dehydrogenase (LDH) catalyzes the reversible oxidation of lactate to pyruvate. It is an almost universal enzyme found in all types of tissue including muscle, kidney, liver, brain, pancreas, bone marrow and lung. Many types of tissue damage and disease will increase serum LDH levels. LDH activity is best measured by observing the appearance of the coenzyme NADH spectrophotometrically with lactic acid as the substrate.

Fractionation of the various isoenzymes of LDH by agar gel electrophoresis (Zimmerman and Seeff 1970) greatly enhances the

usefulness of the test. The specific isoenzymes identify the damaged organ of origin of the raised LDH in situations where GOT, GPT and other enzyme activities are all elevated. Cornish et al. (1971) reported the identification of serum LDH isoenzyme patterns in serial samples after treating rats with mercuric chloride. The early samples taken one half to four hours after treatment showed increased levels of LDH<sub>5</sub> and slight increases of LDH<sub>4</sub> characteristic of liver damage. The later samples showed marked elevations of LDH<sub>1</sub> and moderate elevations of LDH<sub>2</sub> characteristic of kidney damage. When isoenzyme patterns are examined in a study, serial blood samples should be taken, since a single sample may be misleading if it is not collected at or near the time of maximum tissue release for the isoenzyme being examined. Representative studies examining LDH activities in the rat and rabbit are shown in Table C-1.5 of Appendix C.

#### 4.1.7 Additional Enzymes

Following are brief descriptions of additional useful enzymes that may be, or have been determined in toxicity testing but which are not commonly included in routine testing at this time. As with other biochemical tests for hepatotoxicity, many of these additional tests have clinical utility, which enhances their value in relating animal toxicity data to man. Methods of determination are similar to those used for the more frequently determined enzymes. These essentially consist of analysis for disappearance of a substrate or

appearance of an end product. Sensitivities are enhanced by using radiolabeled substrates specific for the enzyme under investigation. It should be noted that most of the following tests are being used in research and in special studies. They may be better than those used at present, but laboratories tend to retain tests because of their long familiarity and historical values, in spite of reported better results.

Malate Dehydrogenase (MD)

This enzyme catalyzes the reversible oxidation of malate to oxaloacetate. MD is present in the cell cytosol, but is present in greatest quantities in the mitochondria, which may account for its slow or prolonged release after cell damage. Cytoplasmic enzymes are usually released more rapidly than those from mitochondria. Serum MD activity rises after hepatic cell necrosis (Zimmerman and Henry 1979c), but does not rise much after myocardial damage (Zimmerman and Seeff 1970). It increases to a relatively higher level than do LDH isoenzymes for the same amount of necrosis and can be useful in special situations where effects on bone, kidney or pancreas are not involved (Zimmerman and Henry 1979c). It is used also when LDH isoenzymes are not determined. Studies examining MD activity in the rat and rabbit are shown in Table C-1.6 of Appendix C.

Isocitrate Dehydrogenase (ICD)

This enzyme catalyzes the conversion of isocitric acid to alpha-ketoglutarate and is mainly a mitochondrial enzyme. Levels of

activity are high in acute hepatic necrosis, as from heavy doses of carbon tetrachloride; but ICD is not more specific than GPT (Zimmerman and Henry 1979c) and it is only slightly elevated for cirrhosis and obstructive jaundice. It has potential for being a specific test for acute hepatic necrosis. Table C-1.7 of Appendix C shows studies where ICD activities have been measured.

#### Serum cholinesterase (CHE)

The cholinesterase of the serum (CHE) has been referred to as a "pseudocholinesterase", to distinguish it from the "true" cholinesterase (AcCHE) that is found in the erythrocytes and nerve cells.

CHE rapidly hydrolyzes acetylcholine and other cholinesters. This enzyme is included here because its level falls when parenchymatous liver disease--such as hepatitis, hepatic congestion, cirrhosis with jaundice, ascites or other evidence of parenchymal damage--are present (Zimmerman and Henry 1979c; Cutler 1974). It seems to be related to low serum albumin levels, and both seem to be related to depressed protein synthesis in the liver. It is not a more sensitive index of parenchymal function than some other more common enzymes (Cutler 1974). Serum cholinesterase can also be depressed by organophosphate pesticides and related chemicals independently of liver injury. The differential interpretation of decreased CHE levels requires additional tests such as GGT (Zimmerman and Seeff 1970). Table C-1.8 of Appendix C lists a few representative studies where CHE activities have been measured.

Aldolase (ALD) and Phosphohexoisomerase (PHI)

These glycolytic enzymes, are found in all tissues. Aldolase converts fructose-1-6-diphosphate to glyceraldehyde-3-phosphate, and PHI catalyzes the reversible conversion of glucose-6-phosphate to fructose-6-phosphate. Both are markedly increased in toxic hepatitis, but only slightly in obstructive jaundice or cirrhosis. They are thus useful in differential diagnosis of liver damage (Zimmerman and Seeff 1970).

Marked increases of ALD and PHI have been observed in laboratory animals with hepatic necrosis (Korsrud et al. 1971, 1973; Zimmerman et al 1965a, 1965b)(See Tables C-1.9 and C-1.10 in Appendix C). LAD and PHI may also be elevated in rats when liver tumors are present.

Leucine aminopeptidase (LAP)

This peptidase is in the same class as GGT and other transpeptidases (i.e., those enzymes that act on polypeptides or peptide chains freeing amino acids) and has been used in laboratory animal studies. It rises in serum from hepatobiliary diseases, especially in obstructive biliary conditions (Ideo et al. 1972; Clampitt 1978) and follows rises in ALP, GGT and 5'-N (5'-nucleotidase) (Zimmerman 1978). Several LAP isozymes have been identified. LAP, GGT and 5'-N are all useful in animal studies and are not affected by the age of the animals. Studies where LAP activities have been measured are shown in Table C-1.11 of Appendix C.

### 5'-Nucleotidase (5'-N)

This esterase, like the phosphatases, was advocated as a distinguishing test between obstructive and hepatocellular jaundice (Zimmerman and Henry 1979c). Its levels of activity follow LAP and ALP, and the highest levels are found in post-hepatic jaundice. Lower levels are found in parenchymal hepatic dysfunction. In some instances it has been used as a more sensitive test for hepatobiliary disease than ALP (Zimmerman 1978).

## 4.2 Metabolic Tests

The liver performs numerous metabolic functions, including oxidation of fatty acids; formation of lipoproteins, cholesterol and phospholipids; deamination of amino acids; formation of urea; and storage of many substances such as glycogen, vitamins and iron. Alterations in any of these metabolic or storage functions may indicate liver damage. Tests which monitor the three principal types of liver metabolism (i.e., carbohydrate, lipid and protein metabolism) will be described in the following sections. Tests which monitor xenobiotic metabolism will also be discussed.

### 4.2.1 Carbohydrate Metabolism

In mammals, almost all digested carbohydrates are metabolized to glucose, the primary energy source of the body. The liver removes excess glucose from the blood and stores it as glycogen; then returns it to the blood as needed. Glucose levels in the smaller experimental animals are influenced by so many variables that these levels are

of value only as a very general measure of over-all hepatic function when conducted under carefully controlled conditions. The variables affecting blood glucose levels in higher animals have been reported by Benjamin and McKelvie (1978).

Hypoglycemia--lower than normal blood glucose--is a result of decreased hepatic gluconeogenesis. It is associated with increased insulin; starvation; prolonged use of alcohol; toxic chemicals such as sulfonylureas, phosphorus, salicylates, sulfonamides, and propanolol; various tumors; pituitary and adrenal hormone suppression, and diffuse liver disease. Thus, hypoglycemia is not specific for liver damage, but may be due to pathological conditions outside of the liver, such as hyperinsulism from pancreatic-islet cell tumors or hyperplasia (Benjamin and McKelvie, 1978). Hyperglycemia--elevated blood glucose--can be caused by a number of conditions, including hormonal abnormalities, but these are normally not found in experimental animals.

The hexokinase method for blood glucose (Neeley 1972), which uses hexokinase-glucose-6-phosphate dehydrogenase, is accurate and specific and is recommended for use in animal laboratories by Howanitz and Howanitz (1979).

The measurement of blood glucose levels in experimental animals is used only to a limited extent in research studies (See Table C-2 in Appendix C), principally to assess general health, and is not recommended for short-term screening tests because of its lack of specificity.

#### 4.2.2 Lipid Metabolism

Lipids comprise approximately 10 percent of the mammalian body weight and are the most concentrated source of energy in the body. The liver is involved in many important aspects of lipid metabolism including: (1) beta oxidation of fatty acids; (2) formation of lipoproteins; (3) formation of cholesterol and phospholipids; and (4) formation of fat from carbohydrates and proteins. Damage to the liver may disrupt any of these functions. Consequently, monitoring malfunction in liver lipid metabolism can provide a basis for assessing the hepatotoxic effects of test substances (See Table C-3 in Appendix C). Alterations in plasma cholesterol and bile acids are the most frequent indicators of hepatotoxicity.

Cholesterol is both absorbed from digested food in the intestine and produced in body tissues, notably the liver. The liver is the principal source of cholesterol, and also the principal organ for its disposal (Guyton 1976). In mammals, most of the cholesterol is converted to bile acids, which promote the digestion and absorption of fats from ingested foods. Cholesterol is also a precursor in the synthesis of adrenocortical hormones. In liver malfunction, such as hepatitis, hepatocellular jaundice, or cirrhosis, cholesterol levels may be markedly depressed. In cases of post hepatic jaundice or intrahepatic cholestasis, serum cholesterol levels are elevated (Zimmerman 1979a).

There are many methods available for estimating total serum cholesterol. Perhaps the most rapid and convenient method is that of Pearson et al. (1953), which does not require saponification of the cholesterol esters in the serum. Color is developed directly in approximately 20 minutes using p-toluenesulfonic acid which can then be measured in a colorimeter or spectrophotometer at 550 m<sup>μ</sup>. There are also gas-liquid chromatographic and enzymatic methods available; many of which can be automated. In the enzymatic methods, the cholesterol esters are hydrolyzed with cholesterol-ester hydrolase; the cholesterol is then oxidized with cholesterol oxidase and the hydrogen peroxide formed is quantitated colorimetrically. Other sterols will interfere with this method, but bilirubin and hemoglobin will not. It is difficult to compare the cholesterol values obtained by different methods because of the different method sensitivities and possible interfering substances. Consequently, a statement of normal cholesterol levels in various animal species is currently not practical because different methods have been used by different investigators. However, most procedures may be used to indicate changes from levels in normal control animals.

Most of the cholesterol that circulates in the plasma is esterified. Since this esterification process takes place in the liver, when the liver is damaged, a marked decrease in plasma cholesterol esters and a corresponding increase in free cholesterol may be

observed. This cholesterol/cholesterol ester ratio is readily measured and provides a useful indicator of liver dysfunction (Corning 1980).

In most mammals, cholesterol is converted to bile acids. The bile acids are classified as primary and secondary. The two primary acids are cholic--a trihydroxy--and chenodeoxycholic--a dihydroxy bile acid. Considerable variation among species has been found in the levels and ratios of these two acids (White et al 1968). They are conjugated with glycine and taurine in hepatic cells, and are actively transported into the gall bladder, where they are stored in the bile until food enters the digestive tract. The bile, with its bile acids, is then discharged into the small intestine, where it is available for use in digesting fats.

The secondary bile acids are formed by bacterial and other action in the intestine. The most important of the secondary acids are deoxycholic and lithocholic acids, some of which are excreted in feces. The normal liver cells are very efficient in taking the bile acids from the portal blood, into which they have been absorbed from the intestinal tract, and re-excreting them into the bile. This insures a low level of bile acids in peripheral blood, although a relatively large amount is present in the biliary system, portal vein and intestine.

Because of the active reabsorption, pre- and post-prandial blood levels of endogenous (or administered) bile acids can act as a good

liver-function test. Comparisons of pre- and post-prandial blood bile acid levels indicate the speed (efficiency) of the liver cells in excreting the surge of primary and secondary bile acids absorbed into the blood after a meal. It follows that the administration of bile acids, and the determination and comparison of pre- and post-administration blood bile acid levels, also demonstrate liver bile acid clearing ability. The latter procedure (bile acid administration) is more easily controlled in laboratory animal studies than examining blood bile acid levels before and after animal feeding.

Anwer et al. (1976) studied plasma bile acid levels using enzyme methods in dogs and other domestic animals (i.e., sheep, calves, and ponies), all of mixed breeding, before and after the production of liver damage by carbon tetrachloride. They found significant increases in the concentration of the bile acids as well as of bilirubin (except in dogs), ID, GOT, and GPT in all animals. They concluded that bile acid levels could be used to test liver function in experimental animals.

Unfortunately, the methods currently available for measurement of total serum bile acids and of the individual bile acids are too difficult and time-consuming for routine application in a screening program, except possibly at more advanced levels of investigation. The three methods usually employed are: (1) enzymatic hydroxysteroid dehydrogenase assay, (2) gas-liquid chromatography, and (3) radioimmunoassay (Zimmerman 1979a). The gas-liquid chromatography or the

enzyme methods are better adapted to animal testing than the immunoassay method because of the complications involved in preparing species-specific antigens for each type of animal tested.

#### 4.2.3 Protein Metabolism

Protein is produced by the human liver at a rate of up to 4 grms/hr (Guyton, 1976) in the endoplasmic reticulum of the hepatic parenchymal cells. The site of protein synthesis is the rough endoplasmic reticulum (Miyai 1979). Some of these proteins are transferred to the Golgi complex, secreted into the space of Disse, and eventually enter the plasma pool. Thus, chemical damage at almost any structural level of the liver, from damage to subcellular components on up to generalized necrosis, can affect the protein metabolizing functionality of the liver.

There are three major components of plasma proteins: albumin, globulin and fibrinogen. Their concentrations can be affected by chemical injury to either the liver or the kidney, and by malnutrition (See Table C-4 in Appendix C). Abnormal serum protein levels can arise from anorexia in short-term testing with experimental animals due to their refusal to eat chemically treated food, malabsorption due to chemical action, or toxic effects of the test material on the kidneys or liver.

Methods for measuring total protein, albumin and globulin in plasma and other body fluids are numerous. They are of three different types: protein-dye binding, electrophoretic mobility, and

immunochemistry. Toluidine blue or Evans blue are utilized because they bind readily to albumin, but their use may result in erroneously low readings, especially in albumin determinations, since the albumin may already have substances bound to it that limit dye uptake. A bromcresol-green dye method for protein determination has been adapted to the autoanalyzer (Davidson et al. 1979).

Electrophoresis can be used to separate not only the three major kinds of circulating proteins, but can also separate protein subgroups. A simple electrophoretic method for animal laboratories was recommended by Street (1970). Separation times were 16 minutes for dog plasma, and 20 minutes for pig and human plasma. With rat serum, it is necessary to use a Barbitone-Acetate buffer system in order to separate the globulins from the albumin. These tests are useful in human and animal research, but have not yet been well enough developed for routine use in short-term toxicity testing. The major difficulty in such development is that each protein in each species is immunologically, and often electrophoretically, different (Davis et al. 1973).

Prothrombin, vitamin K and other clotting factors are either produced (prothrombin) or stored (vitamin K) in the liver. Many liver diseases such as cirrhosis and jaundice, and various chemicals, interfere with clotting because of their effect on the liver cells. Measurement of that function (clotting time) indicates liver cell damage. The test usually used is Quick's one-stage prothrombin time,

wherein the time required for the experimental blood to clot is compared with a normal (control) of the same species at the same time. This test has not been used regularly in animal testing for the detection of liver damage; however it has been utilized in some specialized studies of liver dysfunction and metabolism.

#### 4.2.4 Xenobiotic Metabolism

Hepatotoxicity of a test chemical can be measured by (a) prolongation or enhancement of the physiological effects of a concomitantly administered drug, or (b) decreased rate of metabolism of a marker chemical. Either in vivo or in vitro techniques are available (Akin and Norred 1978; Becker and Plaa 1965; Cagen and Gibson 1977; Chow and Cornish 1978; Plaa 1974, 1975b, 1976).

Measurements of the duration of hexobarbital sleeping time, pentobarbital sleeping time, and zoxazolamine-induced paralysis can be used to measure the functional status of the drug-metabolizing microsomal mixed-function oxidase system (See Table C-5 in Appendix C). These measurements are based on behavioral responses (e.g., restoration of consciousness or motor function). The tests may indicate induction, activation, or inhibition of the microsomal enzymes. For example, prolongation of sleeping time induced by a dose of a barbiturate may be an indication of liver tissue damage by the test compound, or it may indicate a decrease in the rate of metabolism of the administered barbiturate by inhibition of the microsomal enzyme system (Plaa 1975b). Nonetheless, the prolongation of barbiturate

sleeping time is a good measure of hepatic damage when this procedure is properly standardized and correlated with other indicators of liver function (e.g., serum transaminase activity and BSP clearance) (Plaa 1974, 1975b).

The liver has a highly developed capacity to metabolize exogenous compounds. The products of metabolism may be more toxic or less toxic than the original compound. Abnormalities in the metabolism of known foreign substances can provide an indication of liver damage. Detoxification of sodium benzoate or benzoic acid (Cutler 1974) has been used in this context in the animal laboratory as an indication of hepatic damage (See Table C-5 in Appendix C). The benzoate ion is detoxified by conjugation with glycine to produce hippuric acid, which is excreted in the urine.

The procedure for measuring hippuric acid excretion involves administering a standard dose of benzoic acid orally, or sodium benzoate intravenously, and measuring the amount of hippuric acid excreted in the urine in a specific period of time. The percent of recovery of benzoate as hippuric acid provides a two-fold measure of liver function; it measures the presence of a functional enzyme system for conjugation, and an adequate supply of glycine (Byrne 1977a). Significantly decreased urinary hippuric acid is indicative of liver damage. Cutler (1974) reported that the benzoate/hippuric acid excretion test is one of the most sensitive tests for detecting hepatic damage in rats. However, hippuric acid excretion may not be a

valid test for liver function in those laboratory animal species (e.g., dog) that have a different metabolic scheme for the metabolism of benzoate than the rat. For example, in the dog, the predominant metabolite is benzoyl glucuronide and not hippuric acid. Also, in the presence of severe kidney damage, inaccurate results may be obtained. Difficulties in obtaining urine samples from small animals at fixed time periods have somewhat restricted the routine use of this test for short-term toxicity testing (Street 1970).

#### 4.3 Serum Metals

Abnormal serum values for certain metals have been observed in animals and humans with specific hepatic diseases or damage. Liver cells store iron from the breakdown of hemoglobin as ferritin, which is recycled into hemoglobin. Injury to the hepatocyte releases this stored iron. Elevated levels of serum iron have been observed in animals with acute hepatic necrosis and in humans with viral hepatitis (Zimmerman 1979a).

Elevated serum and tissue levels of "free" copper have been observed in humans with Wilson's disease (hepatolenticular degeneration), and decreased serum levels of zinc have been observed in individuals with alcoholic cirrhosis. The use of serum metal levels in detecting hepatic damage has had only limited clinical application in this country (Zimmerman 1979a), and very limited use in animals, therefore, it would not be useful at this time in a routine screening program for hepatotoxic substances.

#### 4.4 In Vitro Techniques

Several procedures have been developed enabling investigators to study biochemical reactions in isolated organs or organ parts without the complicating factors of interference from products of other organs or contents of the blood. These in vitro procedures are frequently used in liver biochemical studies and have been used to screen a limited number of toxic agents (See Appendix D). Whole liver, liver tissues, cells or organelles are cultured in media containing the necessary nutrients to maintain their functions. Their longevity is limited, depending upon the culture techniques used.

The in vitro techniques are especially useful in research studies on the metabolic phases of various products of cell activity. They save time and animals, since one liver can furnish many slices or cells for the study of several substances, as well as provide uniform test material. However, Fry and Bridges (1979) in discussing the value of the in vitro techniques in toxicity and metabolism investigations, cautioned that although the in vitro studies extend our knowledge of metabolic and toxic effects, they can be misleading. As an example, they point out that the rate at which substances enter and exit cells cannot be accounted for; that most of these tissue preparations survive for only one half to two hours; and that all observations must be made during that interval. Also, they suggest that lytic enzymes released from cells may destroy the very substances which are being measured. Protein and lipid binding may also

be over- or under-estimated, and the conjugating systems are usually non-functional in these preparations. The following sections describe the in vitro techniques used in liver toxicity studies, their advantages and disadvantages, and their potential application to a short-term screening program.

#### 4.4.1 Liver Slices

Slices of liver weighing about 50 mg are removed immediately after sacrifice from a normal animal (usually rat), or from an animal that has been pretreated with a test substance. The slices are placed in a container of Ringer's solution and incubated for periods of 5 to 60 minutes. Slices not already exposed to a test substance can be treated by adding the substance to the bath; accelerators or inhibitors of the test substance may also be added. The incubation is then continued for a specified interval and the bath solution is analyzed by standard methods for the presence of indicator compounds such as enzymes, lipids, or other products. For example, Dujovne et al. (1968) treated one of three groups of liver slices with either promazine, chlorpromazine or nothing. They found that both GOT and GPT were present in the chlorpromazine, but not in the promazine or control baths, indicating that the transaminases were released by chlorpromazines. This agreed with the results of whole-animal toxicity testing of the same substances--that is, the transaminases rose in rat serum after chlorpromazine administration. However, all in vitro results cannot be interpreted this simply. The influence of

all the other body tissues and fluids is a necessary part of the whole body process. The liver slice technique does, however, add to the value of other test results. This method is more properly a research tool than a routine screening procedure (See Table D-1 in Appendix D), although, of all the in vitro tests, it is probably the simplest to use and provides the most information on one or two specific enzymes or metabolites. It may be used in advanced levels of a screening program where mechanisms of damage are being investigated. Nevertheless, the results obtained from tissue slices will not necessarily be the same as results obtained from the treatment of the intact animal.

#### 4.4.2 Perfusion Techniques

Much more apparatus and skill are needed for perfusion testing than for liver slices. The whole organ (liver) is removed from the animal (usually rat), and the portal vein and bile ducts are cannulated. This permits fluids to be perfused through the organ via the blood vessels and collected for analysis. The bile can be drained off through the bile duct, to be used for analysis of metabolites or other contents. The preparation is placed in an incubation chamber with a bath containing proper electrolytes and oxygen pressure. Toxic agent effects of a test substance on bile flow, dye elimination, lipid secretion or enzyme activities can be determined by adding chemicals to the fluid being perfused through the liver, collecting the perfusate for analysis; and measuring bile flow in the

common bile duct. An example of the use of perfusion techniques is the work reported by Abernathy et al. (1978). Dantrolene sodium (a drug used to control spastic muscle contraction in humans), when added to rat liver perfusate, inhibited excretion of both ICG and BSP.

The perfusion techniques are principally used in research (see Table D-1.1 in Appendix D) and are too involved to be used in a short-term screening program, except at advanced levels (Level III).

#### 4.4.3 Isolated Hepatocytes

Mammalian liver cells can be isolated by digestion of tissue with collagenase. Viable isolated hepatocytes have been obtained from mice, rats, hamsters, guinea pigs, rabbits, ferrets, dogs, sheep, monkeys, and man (Fry and Bridges 1979). The isolated hepatocytes are then used in suspensions or as monolayer cultures. The hepatocyte cultures remain viable and functional for up to 10 days with appropriately selected culture media, and are used primarily for metabolic studies. They have also been used in monitoring the cytologic and genetic changes from xenobiotic-induced toxicity. Toxicity is commonly measured as the leakage of cytoplasmic enzymes from hepatocytes (Abernathy et al. 1978). Many of the events involved in toxic responses in vivo when animals have been exposed to a hepatotoxin, have also been observed in vitro in isolated hepatocytes exposed to the same substances. For example, hepatocytes exposed to carbon tetrachloride or bromotrichloromethane, lipid peroxidation

and other alterations in membrane lipids have been observed (Weddle et al. 1976). These are the same changes that have been observed in in vivo studies using these two substances. The use of isolated hepatocytes in screening chemical substances has the additional advantage of making it possible to assess a large number of compounds using a single liver cell population.

Several metabolic and functional adjustments can occur in hepatocytes as the cells establish themselves in monolayer culture. This loss of specialized metabolism and function (i.e., dedifferentiation), as the cells are established and time in culture increases, can limit the usefulness of the cultures in studying toxicity. If primary hepatocyte cultures are used, they should be maintained in a nondividing state as normally occurs in vivo, and should not be manipulated to divide and produce cell lines (Fry and Bridges 1979). Varying degrees of dedifferentiation have occurred in these dividing cell lines, and their functions may be very different from in vivo hepatocytes. Any cell trauma that occurred during isolation can be repaired during culture, and therefore this trauma would not be interpreted as a result of test substance exposure.

Isolated hepatocytes in suspension should provide a more useful model system in screening substances for hepatotoxic potential than cell cultures. Suspensions are relatively easy to use and are not affected by dedifferentiation as are cell cultures. On the other hand, the primary hepatocyte culture system is one of the best

studied to date, and even though it is more involved than isolated suspensions, it could be used in a screening program. An outline of studies using hepatocyte cultures is shown in Table D-1.2 of Appendix D.

#### 4.4.4 Tissue Homogenates and Organelles

Liver tissue homogenates have been used in the past to study the adverse effects of agents on liver tissue that could not be studied conveniently in vivo. For example, uptake of radiolabeled amino acids has been examined to demonstrate the effects of toxic chemical injury on protein synthesis. The mammalian liver contains at least 14 different cell types, so homogenates may contain a significant fraction of nonhepatocyte cell types, although the proportion of cell types should be similar to the original tissue. This lack of uniformity in homogenates may be a disadvantage in using them as model systems. Accordingly, homogenates are primarily used in research, and are not used much for short-term screening purposes (See Table D-1.3 in Appendix D).

Centrifugation of either isolated cells or homogenates can be used to isolate subcellular organelles from the hepatocytes. The effects of toxic agents on isolated ribosomes, mitochondria, lysosomes, plasma membranes, Golgi apparatus, nucleoli and nuclei have been studied (Zimmerman 1978). For example in examining the toxic effects of fluoroacetate, Kostyniak et al. (1978) studied liver de-fluorination activity by incubating fluoroacetate with a subcellular

fraction of rat liver. Control fractions were boiled to stop subcellular activity. They compared the amount of ionic fluoride present in the media of the two-cell fraction groups at the end of the incubation period and found there was more fluoride present in the living subcellular fraction medium than the boiled controls. Isolated organelles have only been used in research applications and are not suitable for use in a screening program. Tables D-1.4 through D-1.6 of Appendix D show some representative studies where isolated organelles have been used in research applications.

#### 4.5 Summary

Biochemical tests are sensitive indicators of hepatotoxicity and also provide information concerning the mechanisms of damage to the liver. Biochemical tests for liver damage may be grouped into two broad categories: (1) direct measurements of the activity of specific serum enzymes (e.g., serum glutamic-pyruvic transaminase) and (2) measurements of normal end products of liver function, such as plasma bile acids. Usually, these tests are done in vivo, but may be accomplished in vitro using whole organs, organ slices, isolated hepatocytes, homogenates or isolated organelles.

Damage to the liver by toxic chemicals may be manifested by a release of cellular enzymes into tissue fluids or by impairment of normal enzyme formation. Although the liver is replete with enzymes only a few are useful at this time for short-term toxicity testing of chemicals. Those enzyme tests which are utilized must be carefully

interpreted to be sure than any changes observed are indicative of hepatic damage rather than being a normal response to a metabolic load, or indicative of damage to some other tissue.

The simultaneous measurement of the two transaminases, glutamic-pyruvic transaminase (GPT) and glutamic-oxalacetic transaminase (GOT) gives greater credence to the interpretation of experimental results than the measurement of either enzyme alone. GOT may come from tissues other than the liver, while GPT comes almost exclusively from the liver; the magnitude of changes from effects of xenobiotics is greater for GPT.

Alkaline phosphatase (ALP) is one of the various phosphatases present in liver cells, in addition to being present in many other tissues. Serum ALP determinations are most useful in animal studies for detecting hepatobiliary obstruction.

Gamma-glutamyl transpeptidase (GGT) is found in the liver, pancreas and kidney. Since the smooth endoplasmic reticulum of the liver is the source of most of the serum GGT, GGT is a useful enzyme for detecting hepatic damage and may be a better indicator of dysfunction than the transaminases or ALP.

Ornithine carbamyl transferase (OCT) is only found in the liver where it is involved in nitrogen metabolism. It catalyzes the reversible conversion of ornithine to citrulline, which is a step in the formation of urea. It should be considered seriously for use in animal testing following further development of the analysis techniques because its presence in serum is highly specific for liver injury.

Iditol dehydrogenase (ID) is involved in carbohydrate metabolism in the liver by catalyzing the reversible reaction of sorbitol to fructose. Its presence in serum at elevated levels is a relatively specific indicator of liver injury even though minimal amounts are normally found in serum as well as in semen and skeletal muscle.

Lactic dehydrogenase (LHD) isoenzymes are found in many types of tissue; however, the liver contains principally LDH<sub>4</sub> and LDH<sub>5</sub>. Increases in serum LDH<sub>5</sub> are specific for liver damage and can be useful in differentiating liver damage from damage to other organs having LDH isoenzymes.

Some other enzymes, such as malate dehydrogenase (MD) and isocitrate dehydrogenase (ICD), may also be useful in detecting hepatic dysfunction and damage. Some may even be better than the previously described enzymes; however, they have not been used as frequently and are not as well developed.

In mammals, absorbed carbohydrates are generally metabolized to glucose. The liver converts blood glucose to liver glycogen which is stored until there is a demand for an elevation of blood glucose. Concentrations of glucose in the blood are a general measure of liver function and damage by xenobiotics. However, this is not a sensitive measure of injury, nor is it specific for damage to the liver.

The liver also metabolizes lipids, including the production, excretion and recycling of cholesterol and bile acids. Plasma cholesterol concentrations are not consistent enough in animals to

make them a useful measure of hepatotoxicity. The measurement of the cholesterol/cholesterol ester ratio, however, may be useful. Plasma bile acids are sensitive enough to be considered for short-term testing; unfortunately, the procedures for isolation and analysis are too difficult and time-consuming for most short-term screening programs.

Protein metabolism is an important function of the liver; however, there are no simple tests for monitoring albumin, globulin, or total protein which are sufficiently sensitive or specific to be useful in routine screening. Prothrombin, vitamin K, and other clotting factors are either produced or stored in the liver. The monitoring of blood-clotting time to indicate hepatic dysfunction has not been regularly reported in animals and is not considered useful for routine screening at this time. Alterations in liver xenobiotic metabolism have been useful in assessing liver damage. The most useful have been barbiturate sleeping time and benzoate metabolism to hippuric acid. The benzoate/hippuric acid excretion test is a relatively sensitive measure of hepatic function; however, it has not been used much in the last few years in the routine screening of hepatotoxic substances.

Several in vitro model systems have been developed to be used in liver biochemical studies. These systems have been especially useful in metabolic studies. Most of the biochemical parameters previously described can be examined in the in vitro systems. Isolated hepatocyte suspensions are currently the most useful of the model systems

for short-term screening, although primary hepatocyte cultures are also very useful in assessing hepatotoxic potential. The other in vitro model systems are used for specialized applications and may be useful in determining the mechanisms of toxicity.

## 5.0 CONCLUSIONS AND RECOMMENDATIONS

The testing techniques for the assessment of hepatotoxicity have been classified in three categories: morphologic, functional, and biochemical. The tests included in each of the three categories are shown in Table 5-1 and were categorized on the basis of either their structural or physiological characteristics.

The liver is an organ of diverse functional activity. It performs many metabolic functions and is the principal detoxification organ in the body. Many measurements of hepatic function have been made using a variety of techniques; however, few of these have been found to be useful in detecting and quantifying liver damage. The development of liver functional testing has followed the development of new knowledge concerning the biochemical mechanisms of the liver. For this reason, and because of its essential biochemical functions, most current tests to monitor the liver for damage are biochemical.

The testing of hepatic function in humans is well developed; however, some of the human testing techniques have not been applied to animals. Many of the procedures are not practical for use in laboratory animals. Only those testing techniques that are used in laboratory animals or that could readily be adapted to laboratory animals are described.

Some alterations in liver function may not be indicative of damage. Many tests are sufficiently sensitive to detect biochemical alterations which are a part of natural liver function as the liver

TABLE 5-1  
TESTS USED TO EVALUATE HEPATIC DAMAGE

TEST CATEGORY	SPECIFIC TESTS
Morphological	<ul style="list-style-type: none"> <li>● Gross Inspection</li> <li>● Light Microscopy</li> <li>● Electron Microscopy</li> </ul>
Functional Indicators	<ul style="list-style-type: none"> <li>● Sulphobromophthalein Excretion (BSP)</li> <li>● BSP Transport Maximum (Tm)</li> <li>● Indocyanine Green Excretion (ICG)</li> <li>● ICG Transport Maximum (Tm)</li> <li>● Rose Bengal Excretion</li> <li>● Bilirubin Clearance</li> <li>● Biliary Transport Maximum (Tm)</li> <li>● Bile Flow</li> <li>● Radioactive Colloid Imaging</li> <li>● Radiolabeled Albumin or <math>^{133}\text{Xe}</math> Perfusion</li> </ul>
Biochemical Indicators	<ul style="list-style-type: none"> <li>● Serum Enzyme Activity           <ul style="list-style-type: none"> <li>-Glutamic-oxalacetic transaminase (GOT)</li> <li>-Glutamic-pyruvic transaminase (GPT)</li> <li>-Alkaline phosphatase (ALP)</li> <li>-Ornithine Carbamyl Transferase (OCT)</li> <li>-Iditol Dehydrogenase (ID)</li> <li>-Gamma-glutamyl Transpeptidase (GGT)</li> <li>-Lactic Dehydrogenase Isoenzymes (LDH)</li> <li>-Malate Dehydrogenase (MD)</li> <li>-Isocitrate Dehydrogenase (ICD)</li> <li>-Serum Cholinesterase (CHE)</li> <li>-Aldolase (ALD)</li> <li>-Phosphohexoisomerase (PHI)</li> <li>-Leucine Aminopeptidase (LAP)</li> <li>-5'-Nucleotidase (5'-N)</li> </ul> </li> <li>● Plasma Glucose Levels</li> <li>● Serum Cholesterol Levels</li> <li>● Serum Cholesterol/Cholesterol Ester Ratio</li> <li>● Plasma Bile Acid Levels</li> <li>● Albumin, Globulin and Total Protein</li> <li>● Thymol Turbidity</li> <li>● Cephalin Flocculation</li> <li>● Prothrombin Time</li> <li>● Barbiturate Sleeping Time</li> <li>● Benzoate/Hippuric Acid Excretion</li> <li>● Serum Iron</li> <li>● Serum and Tissue Copper</li> <li>● Serum Zinc</li> </ul>

responds to chemical exposure, but where there are no morphologic changes characteristic of irreversible cytotoxic changes and cell death.

On the basis of available information for the hepatic system, a tiered screening program is recommended for detecting and quantifying hepatic damage in small laboratory animals. Evaluation of individual tests within each category is based on certain considerations. These considerations primarily include: validity of the measurement (e.g., sensitivity, accuracy, reproducibility); costs of measurement (e.g., necessary instrumentation, animals, and labor); the time required to perform the test; and finally, significance with regard to reflecting liver damage. The selection criteria utilized to evaluate the liver tests are described in the following section.

### 5.1 Criteria Used in Evaluating Hepatic System Tests

The following criteria have been selected to evaluate each hepatic system testing technique for inclusion in a short-term screening program:

- state of development sufficient to be reproducible in a screening program
- sensitivity sufficient to detect early subtle forms of damage or to provide an indication of the extent of damage to the system
- procedures and instrumentation sufficiently uninvolved to enable technicians with some additional training to perform the tests, and
- methods sufficiently brief so that each test can be completed within a few days to a few weeks.

Considerations that have also been used to evaluate the tests include (1) the availability of the animals used, and (2) the costs of the test procedures, animals, equipment and maintenance.

The species of animals used for screening affects both the cost and the validity of a particular measurement. The type and the number of animals used to perform an experiment affect the cost not only in terms of the time, but the labor required to perform the test. Also, the sensitivity, accuracy, and reproducibility of a test will depend on the species and number in which the test is performed. However, there are not sufficient data available to establish very many of these relationships with regard to liver tests. Rats are the most common small laboratory animal used for evaluation of the morphological and functional integrity of the hepatic system.

Once the liver tests have been evaluated for suitability in short-term screening, tests which are recommended are subdivided into three levels or tiers based upon the criteria shown in Table 5-2. Investigators should select the tests from each tier that are most suitable for their needs. It is not anticipated that all of the tests recommended in each tier would be used in a screening program. The applicable tests will be selected based upon the screening program protocol design, other experimental observations, and the individual requirements of each investigator.

Those tests routinely used in level I should be simple to perform, inexpensive, quick, and sufficiently sensitive to provide a

TABLE 5-2  
CRITERIA FOR SHORT-TERM  
LIVER TESTING TIERS

<u>Criteria</u>	<u>Level I</u>	<u>Level II</u>	<u>Level III</u>
State of Development	High	Moderate	Moderate to Low
Sensitivity	Moderate	Moderate to High	Moderate to High
Indicates Extent of Damage	Moderate	Moderate to High	High
Complexity of Procedures and Instrumentation	Low	Moderate	Moderate to High
Level of Skill	Low	Moderate	Moderate to High
Test Duration	Hours	Hours to Days	Days to Weeks
Used in Small Animals	Yes	Yes	Yes/No
Cost	Low	Moderate	Moderate to High

good indication of damage to the hepatic system. The tests in level II should be more sensitive than those in level I, and should be better able to describe the extent of damage to the system; however, they are more time-consuming, more difficult to perform, and more expensive than level I tests. The tests in level III are those tests which are not included in level II, but which may be utilized in determining the mechanisms of damage for a particular hepatotoxin. The evaluation of the state of development of tests, the skill necessary to perform them, and the ease with which they are performed, is based on discussions with researchers, and a review of their publications and other literature dealing with liver testing.

#### 5.2 Evaluation of Hepatic System Tests for Application to a Screening Program

The advantages and disadvantages of each testing technique included in the recommendations are described below with a discussion of their potential application to short-term screening. Alternative techniques are described which also could be used. Table 5-3 lists the tests in each level that are recommended for short-term screening. These tests may be used with any mammalian species.

##### 5.2.1 Level I Tests

###### Sulforomophthalein Clearance

Sulfobromophthalein (BSP) is a widely used exogenous dye for monitoring liver function. In more recent years, it has been used less in human clinical medicine, partly because of possible serious adverse reactions, although its use has continued in animals. BSP is

TABLE 5-3

## TESTS RECOMMENDED FOR A TIERED SCREENING PROGRAM

## **Level I**

## Gross Liver Pathology

## Level II

Serum Cholesterol/ Cholesterol Ester Ratio  
Plasma Bile Acids  
Biliary Transport Maximum (Tm)  
Isolated Hepatocyte Suspensions or Monolayer Hepatocyte Cultures  
Light and Electron Microscopy

### **Level III**

Radioactive Colloid Imaging  
Radiolabeled Albumin or  $^{133}\text{Xe}$  Perfusion  
In-Vitro Preparations (Other than those in Level II, e.g., liver  
liver slices of isolated, perfused whole livers)

conjugated in the liver and rapidly excreted. Because it is rapidly eliminated, accurately timed blood samples must be obtained from the experimental animals. Single determination BSP measurements are routinely done in large animals (e.g., dogs), but require venous cannulation in small laboratory animals (e.g., rats). Factors such as hepatic blood-flow changes, extrahepatic disease, cardiac failure, hepatomegaly, fever and shock can affect BSP clearance rates; nevertheless, it is a sensitive and useful technique for assessing liver function and is recommended for use in short-term screening.

#### Indocyanine Green Clearance

Indocyanine Green (ICG) is an exogenous dye that has come into use in the past few years to replace BSP in clinical medicine. ICG has also been used as an alternative to BSP in animals. It is excreted in the bile in an unconjugated form, so it is not dependent on the availability of the hepatic conjugating mechanisms as is BSP. Since ICG is rapidly eliminated in small laboratory animals, accurately timed blood samples must be obtained. ICG is recommended as an alternative to BSP in screening for hepatotoxicity. The investigator may wish to use both BSP and ICG in differential studies utilizing the unique functional characteristics of each dye.

#### Bilirubin Clearance

Bilirubin is present in the serum and originates from the breakdown of hemoglobin in red blood cells. Serum bilirubin includes both conjugated and unconjugated bilirubin. Conjugation occurs in the

liver and is followed by excretion into the bile. Present procedures determine unconjugated and total plasma bilirubin, and the remainder is assumed to be conjugated. Changes in serum bilirubin levels may provide an indication of liver damage in laboratory animals; however, low normal levels of plasma bilirubin in small laboratory animals, such as rats, make small increases difficult to detect. Because serum bilirubin determinations are relatively easy to perform, this test is recommended for inclusion in a screening program.

Urobilinogen in the urine is abnormal and results from increased levels of intestinal bilirubin due to liver damage effects. A simple, semiquantitative technique for detecting urobilinogen in the urine is available using a diazo reagent impregnated "dip stick." Urobilinogen in urine is one of the earliest indications of liver damage and is recommended for inclusion in a screening program. It must be kept in mind, however, that increased hemoglobin breakdown will also increase both serum bilirubin and urine urobilinogen levels.

#### Benzoate/Hippuric Acid Excretion

Hippuric acid recovered from the urine following an administered dose of benzoate provides a measure of liver cell dysfunction and damage. The test is one of the most sensitive tests for detecting hepatic damage in rats. However, it may not be a valid test of hepatic function in laboratory animals such as the dog that do not metabolize benzoate predominantly to hippuric acid. Also, if an animal has severe kidney damage, inaccurate results can occur.

Accurately timed urine samples must be obtained from experimental animals. This requirement limits the use of this test in small laboratory animals. Because this is a sensitive measure of liver dysfunction, it is recommended for inclusion in a screening program.

Barbiturate Sleeping Time

Measurement of the duration of barbiturate sleeping time can be used to measure the status of the drug-metabolizing function in the liver. Prolongation of sleeping time may indicate functional liver damage or simply a reversible decrease in the rate of metabolism of the administered barbiturate by a test substance.

This test is a good measure of hepatic damage when it is properly standardized and correlated with other indicators of liver function (e.g., serum transaminase activity and BSP clearance), and is recommended for inclusion in a short-term screening program.

Serum Enzymes

Serum Enzymes are important in detecting liver dysfunction and disease. They have been used for many years in human clinical medicine and they play a major role in hepatic diagnostic programs. Furthermore, they are useful in detecting liver dysfunction and damage in laboratory animals. When liver cells are either damaged or destroyed, several enzymes are released into serum. Variations in serum enzyme activities may occur in the damaged liver before functional changes are observed because of the functional reserve capacity of the liver; therefore, serum enzymes provide a sensitive

indication of hepatic damage. Serum enzyme determinations are relatively simple, easy to perform, reproducible and inexpensive. The disadvantages are that most serum enzymes are not specific for the liver, but may be found in many other tissues, and that some increases in enzyme levels are due, not to cellular damage, but to increased cellular membrane permeability or increased metabolic activities as a part of normal liver function. For these reasons, serum enzyme determinations should be made in conjunction with other liver function tests.

Four enzymes are recommended for use in Level I of a screening program. These are glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), alkaline phosphatase (ALP), and lactic dehydrogenase (LDH). Other enzymes, such as ornithine carbamyl transferase (OCT), iditol dehydrogenase (ID) and gamma-glutamyl transpeptidase (GGT) could also be included, based on the interests and preferences of the investigator. These enzymes were chosen because they have been the most frequently used in detecting hepatic dysfunction. They are sensitive indicators of liver damage, and considerable information exists concerning their normal levels, fluctuations and variations. It should be noted, however, that GOT, GPT and ALP are less useful in small laboratory animals (e.g., rats) than in large animal species (e.g., dogs) because of the large variability within and among individual animals. For this reason, GGT has been suggested as a substitute for GOT, GPT and ALP in small animals even though it is not as specific for liver injury as GPT.

### Gross Liver Pathology

Gross examination of the liver for size and color should be performed following level I screening. This is an essential aspect of a short-term screening program since the liver is susceptible to enlargement (hepatomegaly) and discoloration (cholestasis, steatosis, cirrhosis and fibrosis) following toxic chemical insult. The gross necropsy may indicate the need for more detailed microscopic study.

#### 5.2.2 Level II Tests

##### Serum Cholesterol and Bile Acids

Cholesterol is absorbed from digested food in the intestine and is produced in body tissues, notably the liver, which is the principal source of cholesterol as well as the principal organ for disposal of cholesterol. Most of the cholesterol that circulates in the plasma is esterified. The esterification process takes place in the liver, so when the liver is damaged there is a marked decrease in plasma cholesterol esters and a corresponding increase in free cholesterol. Variations in cholesterol/cholesterol ester ratios can provide an indication of hepatic dysfunction and cholesterol levels are relatively easy to determine.

In mammals, most cholesterol is converted to bile acids. Monitoring plasma bile acids can provide a good indication of liver dysfunction. The monitoring of both serum cholesterol/cholesterol ester ratios and plasma bile acids is recommended for inclusion in level II of a short-term screening program for hepatotoxicity.

### Biliary Transport Maximum

The bilirubin transport maximum ( $T_m$ ) is determined by infusing sufficient bilirubin to saturate the transport and conjugation mechanisms of the liver. This test is a sensitive measure of hepatic function, even though it is laborious to perform. The use of  $^{14}C$ -labeled bilirubin simplifies the analysis of samples.

### Cultured Hepatocytes

Either isolated hepatocyte suspensions or monolayer hepatocyte cultures may be used, based upon the needs and preferences of the investigator. Both have certain advantages and disadvantages. Viable isolated hepatocytes have been obtained from most common laboratory animals and from man. Once isolated, hepatocytes may be used either as suspensions or in monolayer cultures. Hepatocyte suspensions are relatively easy to work with and they maintain most of their in vivo functional and metabolic properties. Also, a large number of substances can be assessed using a single, uniform cell population. The disadvantages of the suspensions are that they must be used within a few hours after isolation or they begin to deteriorate; and that the isolation procedure can cause cellular trauma, which may be difficult to distinguish from the cytologic effects of the test substance being examined.

Monolayer cultures also have several advantages. They can remain viable for extended periods (days to weeks) if properly maintained. Also, any cell trauma occurring during isolation can be

repaired during culture. The disadvantages of monolayer cultures are that they are more difficult to maintain and treat than suspensions, and they may lose some of their specialized metabolic and functional characteristics as the cultures age. In the past few years, investigators have developed specialized media and culturing techniques to aid in maintaining high levels of differentiation in cultured hepatocytes. The application of these techniques requires a high level of skill to be successful. For these reasons, the isolated hepatocyte suspensions provide a more useful model system in short-term screening than do monolayer cultures.

#### Light and Electron Microscopy

Some degree of morphologic examination is essential in any short-term screening program for hepatotoxicity. The extent of the examination depends upon the needs of the investigator and the purpose of the testing. In a short-term screening program for hepatotoxic potential, gross examination and light microscopy are recommended. These studies are necessary to assess the results from other liver function tests. Electron microscopy is usually reserved for research purposes. It may be used selectively in a short-term screening program for the verification of the results of other tests when a specific mode of action is suspected or where findings from other tests are inconclusive.

### 5.2.3 Level III Tests

#### Radioactive Colloid Imaging

Radioactive sulfur colloids containing radioisotopes, such as gold-198, indium-113m and technetium-99m, are taken up by reticuloendothelial cells and provide for nuclear imaging of the liver.

Areas that fail to accumulate radioactivity or that show diffuse accumulation may represent pathologic processes. This technique may provide useful information to better describe the pathologic processes of a specific hepatotoxic substance.

#### Radiolabeled Albumin or $^{133}\text{Xe}$ Perfusion

Radiolabeled albumin or  $^{133}\text{-Xenon}$  have been infused in the blood and the blood-flow rates measured in the liver. These are specialized techniques to measure hemodynamic parameters in the liver and may provide valuable information concerning the pathologic processes of a chemical substance in a specific region of the liver.

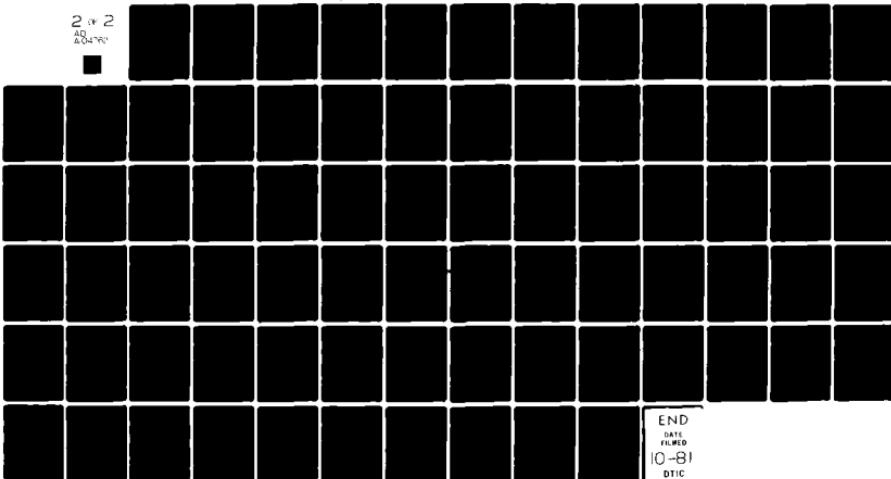
#### In Vitro Preparations

A number of in vitro models are available for the study of hepatic function. The cultured hepatocyte techniques were recommended previously for use in level II. Other techniques, such as the isolated perfused liver and liver slices, are recommended for use in level III because they may provide valuable information concerning the pathologic mechanisms of specific test substances. Biochemical changes can be carefully monitored using these techniques. Many of the functional testing techniques used in vivo have been adapted for use in in vitro model systems.

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**APPENDIX A**  
**MORPHOLOGIC INDICATORS OF HEPATIC DAMAGE**

TABLE A-1  
MORPHOLOGIC INDICATORS OF HEPATIC DAMAGE

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
GROSS MORPHOLOGY, LIVER WEIGHT	MOUSE	ACETONE, AROCLOR 1224, DIMETHYLNITROSAMINE, BARBITURATES, HALOTHANE, METHOXYFLUORANE, CHLOROPHENOTHIAZINE, CHLOROFORM, ALDRIN TETRASUL	SIPES, ET AL., 1978 KUNZ, ET AL., 1967a,b VERSCHURREN, ET AL., 1973	INCREASED LIVER WEIGHT IS USUALLY ACCOMPANIED BY OTHER CHANGES DETECTED BY MICROSOPIC OR BIOCHEMICAL MEANS.
RAT	CARBON TETRACHLORIDE	DIMETHYLNITROSAMINE, DIMETHYLNITRODIETHANOLAMINE, DIMEHTYLTRI-N-SEARINE, ORNTIC ACID, PHENOBARBITAL, SODIUM PHENOBARBITONE, SODIUM SELENATE, TETRASUL, THIACETAMIDE	CLAMPITT, 1978; CORNISH & BLOCK, 1960; CUTLER, 1974; KORSrud, ET AL., 1972 JAECER, ET AL., 1977 KORSrud, ET AL., 1973	
	TETRASUL		CLAMPITT, 1978 MAXFELL, ET AL., 1973 CLAMPITT, 1978 CUTLER, 1974 VERSCHURREN, ET AL., 1973 KORSrud ET AL., 1973	
GUINEA PIG			VERSCHURREN, ET AL., 1973	
RABBIT			VERSCHURREN, ET AL., 1973	
DOG		O,P'-UDT	COPELAND & WANNER, 1974	

TABLE A-1 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
GRASS MORPHOLOGY LIVER WEIGHT	MINISMINE CHICKEN	TETRASUL	VERSCHURREN, ET AL., 1973	
		TETRASUL	VERSCHURREN, ET AL., 1973	

TABLE A-2  
DRUG-INDUCED MORPHOLOGIC CHANGES SEEN BY LIGHT MICROSCOPY

TYPE OF AGENT	HEPATOCELLULAR OR MIXED HEPATOCELLULAR	HEPATOCANALICULAR OR MIXED HEPATOCANALICULAR	CANALICULAR
GENERAL ANESTHETICS	CHLOROFORM TRICHLORETHYLENE HALOTHANE METHOXYFLUORANE FLUOROXENE		
NEURO AND PSYCHO-TROPIC AGENTS TRANQUILIZERS		CHLORPROMAZINE AND RELATED PHENOTHIAZINES ECTYLUREA CHLORIDIAZEPOXIDE DIAZEPAM IMIPRAMINE	
ANTIDEPRESSANTS	IPRONIAZID AND CONGENERS		
ANTICONVULSANTS	AMYTRYPTYLENE DIPHENYLHYDANTOIN PHENYLACETYLUREA AND CONGENERS		
DRUGS EMPLOYED IN RHEUMATIC AND MUSCULOSKELETAL DISEASE AND AS ANALGESICS	CINCHOPHEN ZOKAZOLAMINE IBUFENAC INDOMETHACIN PHENYLBUTAZONE SALICYLATES ACETAMINOPHEN PROBENECID	PROPOXYPHENE	
DRUGS USED IN ENDOCRINE DISEASE OR AS HORMONAL SUBSTITUTES	PROPYLTHIOURACIL CARBUTAMIDE METAHEXAMIDE ACETOHEXAMIDE	METHIMAZOLE THIOURACIL CHLORPROPAMIDE TOLBUTAMIDE	C-17 ALKYLATED ANABOLIC STEROIDS CONTRACEPTIVE STEROIDS ESTRADIOL
ANTIMICROBIAL AGENT	TETRACYCLINE AND CON- GENERS CHLORAMPHENICOL TRIACETYLOLEANDOMYCIN PENICILLIN GRISEOFULVIN PARAMINOSALICYLIC ACID ISONIAZID ETHIONAMIDE PYRAZINAMIDE RIFAMPICIN SULFONAMIDES SULFONES	ERYTHROMYCIN ESTOLATE NOVOBLOCIN RIFAMPICIN ORGANIC ARSENICALS NITROFURANTOIN IDOXURIDINE XENYLAMINE	

TABLE A-2 (CONCLUDED)

TYPE OF AGENT	HEPATOCELLULAR MIXED HEPATOCELLULAR	HEPATOCANALICULAR OR MIXED HEPATOCANALICULAR	CANALICULAR
AGENTS USED IN CARDIOVASCULAR DISEASE	PHENINDIONE PROCAINAMIDE QUINIDINE $\alpha$ -METHYLDOPA NICOTINIC ACID PAPOVERINE	AJMALINE p-AMINOBENZYLCAFFEINE HYDROCHLORIDE	
ANTINEOPLASTIC CHEMOTHERAPEUTIC AGENTS	CAUSE STEATOSIS ACTINOMYCIN D 4-AMINOPYRAZOLO PYRIMIDINE L-ASPARAGINASE AZACYTIDINE AZAURIDINE BLEOMYCIN CHROMOMYCIN CYCLOHEXIMIDE MITOMYCIN N-DIAZOACETYLGLYCINE HYDRAZIDE PUROMYCIN METHOTREXATE (ALSO CAUSES CIRRHOSIS) TETRACYCLINE CAUSE NECROSIS CALVACIN MITHRAMYCIN URETHANE CYCLOPHOSPHAMIDE CHLORAMBUCIL 6-MERCAPTOPURINE	4,4'-DIAMINODIPHENYLAMINE BUSULFAN AZATHIOPRINE	
MISCELLANEOUS AGENTS	TANNIC ACID TRIMETHOBENZAMIDE TRIPELENNAMINE OXYPHENISATIN PHENAZOPYRIDINE	CARBAMAZEPINE	

SOURCE: ZIMMERMAN (1976).

**APPENDIX B**  
**TESTS INDICATIVE OF HEPATIC FUNCTION**

TABLE 8-1 TESTS INDICATIVE OF HEMATOCYTIC FUNCTION

TABLE B-1 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SUDANOPHTHALIN (BSP) CLEARANCE	SHEEP	CARBON TETRACHLORIDE <i>P. LEPTOSPIROMYIA</i>	AL-KHALIDI AND GEMA, 1966 MALARDE ET AL., 1977	

TABLE B-1.1  
TESTS INDICATIVE OF LEPATIC FUNCTION

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
INHAYANINE GREEN (ICG) CLEARANCE	MOUSE RAT	PARAQUAT  ALLYL ALCOHOL. CARBON TETRACHLORIDE	CAGEN AND GIBSON, 1977  KLINGER AND SITTNER, 1976	ICG HAS COME INTO USE IN THE LAST FEW YEARS PARTLY REPLACING BSP. ICG IS RECOMMENDED AS AN ALTERNATIVE TO BSP IN ANIMAL STUDIES.

TABLE B-1.2  
TESTS INDICATIVE OF HEPATIC FUNCTION

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
BILIRUBIN METABOLISM ▲ CLEARENCE	MOUSE	BILE DUCT CANULATION CARBON TETRACHLORIDE $\alpha$ -MAMTHYLISOTHIOCYANATE CONGENERS PHOSPHORUS XANTHOASCIN	PLAA AND BECKER, 1965 BECKER AND PLAA, 1965 CASALS AND OULTSKY, 1946 CASALS AND OULTSKY, 1946 OHTSUBO ET AL., 1976	BILIRUBIN ORIGINATES FROM THE BREAKDOWN OF HEMOGLOBIN IN RED BLOOD CELLS. IT IS CONJUGATED IN THE LIVER AND EXCRETED IN THE BILE. CHANGES IN SERUM BILIRUBIN LEVELS IN LABORATORY ANIMALS MAY PROVIDE AN INDICATION OF LIVER DAMAGE; HOWEVER, LOW NORMAL LEVELS OF PLASMA BILIRUBIN IN SMALL LABORATORY ANIMALS SUCH AS RATS AND MICE MAKE SMALL INCREASES DIFFICULT TO DETECT. BILIRUBIN DETERMINATIONS ARE RELATIVELY EASY TO PERFORM.
RAT	ASPIRIN CARBON TETRACHLORIDE		BALASUBRAMANIAN, ET AL. 1977 BALASUBRAMANIAN, ET AL. 1977 CLAMPITT, 1978; CUTLER, 1977 1974; JUDAH ET AL., 1965; JUGGI, 1977; REES AND SINHA, 1960; SHUCKLER ET AL., 1976	
	ETHANOL ETHIONINE 6-HEXAChLOROCTCLOHEXANE LEAD ACETATE ORTIC ACID PHYTOHEMAGGLUTININS SODIUM SELENATE SODIUM PHENOBARBITAL THIOL BENZAMIDE THIOLBENZAMIDE DERIVATIVES		BALASUBRAMANIAN, ET AL. 1977 SHUCKLER, 1978 TREJO ET AL., 1976 CLAMPITT, 1978 IKEGHMONU AND BASSIR, 1976. 1977 CUTLER, 1976 CLAMPITT, 1978 REES AND SINHA, 1960 HANZLIK ET AL., 1978	DEGLACOMO ET AL., 1977
GUINEA PIG	AMPHETAMINE SULFATE PHENOBARBITAL RESERPINE			

TABLE B- 1.2 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
BILIRUBIN METABOLISM & CLEARANCE	DOG	CARBON TETRACHLORIDE	ANNER ET AL., 1976	
	SHEEP	CARBON TETRACHLORIDE P. <u>LEPTOSTROMIFORMIS</u>	ANNER ET AL., 1976 MALHERBE ET AL., 1977	
	CALF	CARBON TETRACHLORIDE	ANNER ET AL., 1976	
	PONY	CARBON TETRACHLORIDE	ANNER ET AL., 1976	

APPENDIX C  
BIOCHEMICAL DAMAGE INDICATORS

TABLE C-1  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SERUM GLUTAMIC OXALACETIC TRANSAMINASE (SGOT, EC 2.6.2.2)	MOUSE	ALLYL ALCOHOL, $\alpha$ -MANITIN BROMOBENZENE CARBON TETRACHLORIDE COUNARIN	STRUDEL ET AL., 1978 ENDELL AND SEIDEL, 1978	SGOT IS A FREQUENTLY USED TRANSAMINASE ENZYME IN THE DETECTION OF HEPATIC CELLULAR MALFUNCTION. IT IS PRESENT IN MANY DIFFERENT CELLS OTHER THAN LIVER, INCLUDING BRAIN, RED BLOOD CELLS, KIDNEY, SKIN, PANCREAS, CARDIAC AND SKELETAL MUSCLE. PARTICULARLY HIGH GOT CONCENTRATIONS ARE FOUND IN THE LIVER AND MYOCARDIUM. DAMAGE TO THESE TWO TISSUES MAY GREATLY INCREASE SGOT ACTIVITIES. SGOT REMAINS QUITE CONSISTENTLY THE SAME IN MOST LABORATORY ANIMAL SPECIES AND IS RECOMMENDED FOR USE IN ANIMAL STUDIES.
	RAT	ETHANOL D-GALACTOSAMINE PARACETAMOL PHALLOLIDIN PRAESODIUM THIOACETamide  ACTINORYCIN D ASPIRIN BILE DUCT LIGATION CADMIUM CARBON TETRACHLORIDE	BADER ET AL., 1974 BALASURRAMANIAN ET AL., 1977 BASS ET AL., 1978 TIDE ET AL., 1972 COOK ET AL., 1974  ASADA, 1958; ASADA AND GALAMBOS, 1963; BALASURRAMANIAN ET AL., 1967; BASS ET AL., 1978; CLAMPITT, 1978; CORNISH AND BLOCK, 1960; FUJISAMA ET AL., 1976; GRICE ET AL., 1971; IDSO ET AL., 1972; KORSrud ET AL., 1972; KUMATA ET AL., 1975; RAMBOER ET AL., 1972; REES AND SINHA, 1966; SHUCKLER ET AL., 1976; ZIMMERMANN ET AL., 1963a,b  DIETHANOLAMINE DIMETHYLNITROSAMINE	GRICE ET AL., 1971; KORSrud ET AL., 1973 FUJISAMA ET AL., 1976; KORSrud ET AL., 1973

TABLE C-1 (CONTINUED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SERUM GLUTAMIC OXALACETIC TRANSAMINASE (SGOT, EC 2.6.2.2)	RAT (CONTINUED)	ENDOTOXIN ETHANOL. ETHIONINE GALACTOSAMINE $\gamma$ -GLYCOPHOSPHATE $\gamma$ -HEXAACHLOROCYCLOHEXANE LEAD LEAD ACETATE MERCURIC CHLORIDE NITROSAINES OROTIC ACID PARACETAMOL PHYTOHEMAGGLUTININS PRAESODIUM NITRATE SODIUM PHENOBARBITONE THIOACETAMIDE XYLITOL	COOK ET AL., 1974 BALASUBRAMIAN ET AL., 1977; FUJISAWA ET AL., 1976 SMUCKLER ET AL., 1976 GANG ET AL., 1976 RAHJA ET AL., 1977 BALASUBRAMIAN ET AL., 1977 COOK ET AL., 1974 TREJO ET AL., 1972 CRICE ET AL., 1971 NISHIE, 1976 CLAMPITT, 1978 DIXON ET AL., 1975; WILLSON AND HART, 1977; <sup>a</sup> IKEUCHI AND BASSIR, 1976, 1977 VON LEBHANN ET AL., 1976 CLAMPITT, 1978 KORSRUD ET AL., 1973; REES AND SINHA, 1980; SMUCKLER ET AL., 1976 TRUHAUT ET AL., 1977	
	HAMSTER	D-GALACTOSAMINE	JAMES ET AL., 1975	
	RABBIT	CATION TETRACHLORIDE COLLAGENASE	DINMAN ET AL., 1962; FOX ET AL., 1962 BENEDOVA ET AL., 1974	

TABLE C-1 (CONTINUED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SERUM GLUTAMIC OXALACETIC TRANSAMINASE (SGOT, EC 2.6.2.2)	DOG	ADENOSINE BILE DUCT LIGATION CARBON TETRACHLORIDE URANYL NITRATE	MHANALPH ET AL., 1973 TECERIS ET AL., 1969 ANWER ET AL., 1976; TECERIS ET AL., 1969 TECERIS ET AL., 1969	
	SHEEP	CARBON TETRACHLORIDE <i>P.</i> LEPTOSTROMONIUS	ANWER ET AL., 1976 MALHERBE ET AL., 1977	
	CALF	CARBON TETRACHLORIDE	ANWER ET AL., 1976	
	PONY	CARBON TETRACHLORIDE	ANWER ET AL., 1976	
	SQUIRREL MONKEY	CHENOODEOXYCHOLIC ACID	SUZUKI ET AL., 1977	
	RHECUS MONKEY	CHENOODEOXYCHOLIC ACID UROSOODEOXYCHOLIC ACID	FEDOROWSKI ET AL., 1978	

TABLE C-1 (CONTINUED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SEUM GLUTAMIC PYRUVIC TRANSAMINASE (SGPT, EC 2.6.1.2)	MOUSE	ALLYL ALCOHOL α-AMANITIN BROMOBENZENE CARBON TETRACHLORIDE COCAINE DIETHYLENETRIAMINEPENTACETIC ACID ETHANOL FURYLFLUORAMIDE D-GALACTOSAMINE PARACETAMOL (ACETAMINOPHEN) PARQUAT PHALLOIDIN PHOSPHORUS TETRAISUL THIOACETAMIDE XANTHOASCIN	BALAZS ET AL., 1961; STRUBELT ET AL., 1978 HORIUCHI ET AL., 1978; HORNWITZ, 1972; STRUBELT ET AL., 1978 EVANS AND HARBISON, 1978 MORGAN AND SMITH, 1974 STRUBELT ET AL., 1978 HORIUCHI ET AL., 1978 JAMES ET AL., 1975 STRUBELT ET AL., 1978 CAGEN AND GIBSON, 1977 STRUBELT ET AL., 1978 HORNWITZ, 1972 VERSCHUUREN ET AL., 1973 STRUBELT ET AL., 1978 OHTSUBO ET AL., 1976 BALAZS ET AL., 1962 BALASURRAMANIAN ET AL., 1977 IDEO ET AL., 1972 ASADA, 1958; BALASURRAMANIAN ET AL., 1977; BALAZS ET AL., 1961; CLAMPITT, 1978; CUTLER, 1974; FUJISAWA ET AL., 1976; IDEO ET AL., 1972; KORSRUD ET AL., 1972; KUMATA ET AL., 1975; RAMBOER ET AL., 1972; SHICKLER ET AL., 1976; ZIMMERMAN ET AL., 1965a,b BERESOVA ET AL., 1974 JAEGER ET AL., 1977 KORSRUD ET AL., 1973	SGPT IS A FREQUENTLY USED TRANSAMINASE ENZYME IN THE DETECTION OF HEPATIC CELLULAR MALFUNCTION. SGPT IS MORE SPECIFIC FOR LIVER DAMAGE THAN SGOT BECAUSE HIGH CONCENTRATIONS OF SGPT ARE FOUND ONLY IN THE LIVER TISSUE. IN EXPOSURE TO HEPATOXIC SUBSTANCES, SGPT LEVELS TEND TO RISE HIGHER AND REMAIN SO FOR LONGER PERIODS THAN THOSE OF SGOT; HOWEVER, THERE ARE GREATER SPECIES VARIATIONS AMONG SGPT RESULTS. SGPT IS OF CONSIDERABLE VALUE IN DETECTING EARLY HEPATIC DAMAGE AND IS RECOMMENDED FOR USE IN SCREENING STUDIES.
	RAT	COLLAGENASE 1,1-DICHLOROETHYLENE DIETHANOLAMINE		

TABLE C-1 (CONTINUED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SERUM GLUTAMIC PYRUVIC TRANSAMINASE (SGPT, EC 2.6.1.2)	RAT (CONTINUED)	DIMETHYLNITROSAMINE ENDOTOXIN ETHANOL ETHIONINE $\beta$ -GLYCEROPHOSPHATE $\gamma$ -HEXAChLOROCYCLOHEXANE LEAD ACETATE IODIPAMIDE MEGLUMINE ORTIC ACID PARACETAMOL PHOSPHORUS PYRTOHENOAGGLUTININS PIASZODYMUM NITRATE SODIUM PHENOBARBITAL SODIUM SELENATE TETRASUL THIOACETAMIDE THIOPHENZAMIDE DERIVATIVES XYLITOL	BALAZS ET AL., 1961; FUJISAMA ET AL., 1976; KONSRIID ET AL., 1973 COOK ET AL., 1974 BALASUBRAMIAN ET AL., 1977; FUJISAMA ET AL., 1976 ASADA, 1958; BALAZS ET AL., 1961; SHUCKLER ET AL., 1976 RAHJEA ET AL., 1977 BALASUBRAMIAN ET AL., 1977 COOK ET AL., 1976 TREJO ET AL., 1972 BURK AND BARNHART, 1979 CLAMPITT, 1978 DIXON ET AL., 1975 BEN HUR AND APPLEBAUM, 1973 IKEGUCHI AND BASSIR, 1976, CUTLER, 1976 VERSCHUUREN ET AL., 1973 BALAZS ET AL., 1961; JUDAH ET AL., 1965; KONSRIID ET AL., 1973; SHUCKLER ET AL., 1976 HANZLIK ET AL., 1978 TRUHAUT ET AL., 1977 VERSCHUUREN ET AL., 1973 JAMES ET AL., 1975	

TABLE C-1 (CONTINUED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SERUM GLUTAMIC PYUVIC TRANSANINASE (SOFT, EC 2.6.1.2)	RABBIT	CARBON TETRACHLORIDE COLLAGENASE TETRASUL	DINMAN ET AL., 1962; FOX ET AL., 1962 BENESOVA ET AL., 1974 VERSCHUUREN ET AL., 1973	
	DOG	ADENOSINE BILE DUCT LIGATION	BHANALPH ET AL., 1973 TEGERIS ET AL., 1969	
	MINIMINE	TETRASUL CARBON TETRACHLORIDE URANYL NITRATE	VERSCHUUREN ET AL., 1973 ANNER ET AL., 1976; TEGERIS ET AL., 1969 TEGERIS ET AL., 1969	
	SHEEP	CARBON TETRACHLORIDE THIOACETAMIDE	AL-KHALIDI AND GEMA, 1966; ANNER ET AL., 1976 AL-KHALIDI AND GEMA, 1966	
	SWINE	CARBON TETRACHLORIDE URANYL NITRATE	TEGERIS ET AL., 1969	
	CALF	CARBON TETRACHLORIDE	ANNER ET AL., 1976	
	PONY	CARBON TETRACHLORIDE	ANNER ET AL., 1976	

TABLE C-1 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SERUM GLUTAMIC PYRUVIC TRANSAMINASE (SGPT, EC 2.6.1.2)	Rhesus monkey  CHICKEN	CHENOODEOXYCHOLIC ACID POLYCHLORINATED BIPHENYL UNSOODEOXYCHOLIC ACID  TETRASIL.	PEDROWSKI ET AL., 1978 ALLEN ET AL., 1974  VERSCHUUREN ET AL., 1973	

TABLE C-1.1  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
ALKALINE PHOSPHATASE (ALP, EC 3.1.2.1)	MOUSE	TETRASUL MANTHOASCIN	VERSCHUUREN ET AL., 1973 OHTSUBO ET AL., 1976	ALP IS ONE OF THE MANY PHOSPHATASES PRESENT IN LIVER CELLS. IT IS PRESENT IN MANY OTHER TISSUES INCLUDING LIVER, INTESTINE, SPLEEN, BLOOD CELLS, KIDNEY, AND PLACENTA. ITS GREATEST NORMAL LEVELS ARE FOUND IN YOUNG MAMMALS WHERE THERE IS THE GREATEST OSTEOBLASTIC CELLULAR ACTIVITY. FOR THIS REASON, ALP DETERMINATIONS ARE MORE USEFUL IN ADULT ANIMALS.
	RAT	ASPIRIN BILE DUCT LIGATION CARBON TETRACHLORIDE	BALASUBRAMANIAN ET AL., 1977 IDFO ET AL., 1972 BALASUBRAMANIAN ET AL., 1977; CLAMPITT, 1978; IDEO ET AL., 1972; KUMATA ET AL., 1975; REES AND SINHA, 1960	ALP DETERMINATIONS ARE MOST USEFUL IN DETECTING HEPATOCELLULAR AND HEPATOCANALICULAR BILIARY OBSTRUCTION IN LABORATORY ANIMALS.
		COLLAGENASE ETHANOL $\gamma$ -HEXACHLOROCYCLOHEXANE OROTIC ACID PHYTOHEMOAGGLUTININS SODIUM PHENOBARBITONE TETRASUL THIACETAMIDE XYLITOL	BENESOVA ET AL., 1974 BALASUBRAMANIAN ET AL., 1977 CLAMPITT, 1978 IKEUCHI AND BASSIR, 1977 CLAMPITT, 1978 VERSHUUREN ET AL., 1973 REES AND SINHA, 1960 TRUAUT ET AL., 1977	
	GUINEA PIG	AMPHETAMINE SULFATE PHENOBARBITAL RESERPINE TETRASUL	DEGIACOMO ET AL., 1977 VERSCHUUREN ET AL., 1973	
	RABBIT	CARBON TETRACHLORIDE COLLAGENASE TETRASUL	DINMAN ET AL., 1962 BENESOVA ET AL., 1974 VERSCHUUREN ET AL., 1973	

TABLE C-1.1 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
ALKALINE PHOSPHATASE	DOG	CHLOROFORM DISSECTS	KREEF ET AL., 1978 SAINI ET AL., 1978	
	MINI-SWINE	TETRAISOL	VERSHUUREN ET AL., 1973	
	CHICKEN	TETRAISOL	VERSHUUREN ET AL., 1973	

TABLE C-1.2  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
OROTIDINE CARBONYL TRANSFERASE (OCT, EC 2.1.1.3.)	RAT	CARBON TETRACHLORIDE 1,1-DICHLOROETHYLENE	DROTHAN, 1975 JAECER ET AL., 1977	OCT IS A HIGHLY SPECIFIC ENZYME FOR THE LIVER CYTO-FLASH AND IT CAN RISE SPECIALLY IN LIVER CELL NECROSIS; HOWEVER, IN SOME ANIMAL SPECIES SUCH AS THE DOC, OCT POLLOWS TRANSMI-NASE LEVELS. LEVELS OF SUBSTRATE MAY NEED TO BE ADJUSTED FOR DIFFERENT SPECIES.
	GUINEA PIG	33 ORGANIC SOLVENTS	DIVINCENTZ AND KRASAVAGE, 1974	
	MINISWINE	CARBON TETRACHLORIDE URANYL NITRATE	TEGRIS ET AL., 1969	
	DOC	BILE DUCT LIGATION CARBON TETRACHLORIDE CHLOROFORM URANYL NITRATE	CHALIFOUX, 1970 CHALIFOUX, 1970; TEGRIS ET AL., 1969 KEEFE ET AL., 1978 TEGRIS ET AL., 1969	

TABLE C-1.3  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
IBITOL DEHYDROGENASE (ID, EC 1.1.1.14)	MOUSE	ALLYL ALCOHOL, $\alpha$ -AMININITIN BROMOBENZENE CARBON TETRACHLORIDE COUMARIN D-GALACTOSAMINE ETHANOL PHALLOIDIN PHASODIUM NITRATE CARBON TETRACHLORIDE	STRUBELT ET AL., 1978 ENDERL AND SELDEL, 1978 STRUBELT ET AL., 1978 ASADA AND GALAMBOS, 1963; KORSRUUD ET AL., 1972; YAGHMAS AND VILLENEUVE, 1977 JÄGER ET AL., 1977; JÄGER, 1977	ID IS PRESENT IN NORMAL LIVER CELLS AND IN LIMITED AMOUNTS IN SERUM AND SKELETAL MUSCLE. IT APPEARS IN THE SERUM IN LARGE QUANTITIES WHEN THERE IS LIVER CELL DAMAGE. BEYOND BEING SPECIFIC FOR LIVER CELL DAMAGE, IT IS A HIGHLY SENSITIVE INDICATOR OF EARLY LIVER DAMAGE.
	RAT	1,1-DICHLOROETHYLENE DIETHANOLAMINE DIMETHYLNITROSAMINE PHASODIUM NITRATE THIOCETAMIDE CYSTEINE, DIETHYLACETATE, TRICHLOROPROPANE, VINYL CHLORIDE	VON REHMANN ET AL., 1976 VON REHMANN ET AL., 1973 KORSRUUD ET AL., 1973 CONNOLY AND JÄGER, 1978	
	DOG	AFLATOXIN B <sub>1</sub> CARBON TETRACHLORIDE THIACETANSAMIDE	HIMES AND CORNELIUS, 1973 ANNER ET AL., 1976; HIMES AND CORNELIUS, 1973 HIMES AND CORNELIUS, 1973	
	SHEEP	CARBON TETRACHLORIDE	ANNER ET AL., 1976	

TABLE C-1.3 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
TOT. TETRAHALOENASE (ID, EC 1.1.1.4)	CALF	CARBON TETRACHLORIDE	AMER ET AL., 1976	
	PONY	CARBON TETRACHLORIDE	AMER ET AL., 1976	

TABLE C-1.4  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
GAMMA-GLUTAMYL TRANSPEPTIDASE (GGT, EC 2.3.2.1)	RAT	BILP DUCT LIGATION CARBON TETRACHLORIDE OROTIC ACID SODIUM PHENOBARBITONE	IDEO ET AL., 1972 CLAMPITT, 1978; IDEO ET AL., 1972 CLAMPITT, 1978	GGT IS FOUND IN THE LIVER, PANCREAS, AND KIDNEY; ALTHOUGH THE QUANTITY IS GREATEST IN KIDNEY TISSUE, THE ORIGIN OF SERUM GGT IS THE LIVER. IT ORIGINATES IN THE LIVER MITOCHONDRIA AND RESPONDS TO MICROSONAL INDUCING COMPOUNDS. GGT IS A SENSITIVE INDICATOR OF HEPATO-BILIARY DYSFUNCTION AND IS A MORE SENSITIVE INDICATOR OF CHOLESTASIS THAN THE TRANS-AMINASES; HOWEVER, IT IS NOT CONSIDERED USEFUL IN SMALL LABORATORY ANIMALS SUCH AS RATS, MICE AND HAMSTERS BECAUSE OF LOW SERUM CONCENTRATIONS.
	DOG	CARBON TETRACHLORIDE	LUM AND GAMBINO, 1972	
	SHEEP	P. LEPTOSTROMIFORMIS	MALHERBE ET AL., 1977	

TABLE C-1.5  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
LACTIC DEHYDROGENASE (LDH, EC 1.1.1.27)	RAT	CARBON TETRACHLORIDE  DIETHANOLAMINE DIMETHYLNITROSAMINE FREEZING HYDRATROPIC ALDEHYDE OROTIC ACID SODIUM PENUMOBARBITONE THIOACETAMIDE	CLAMPITT, 1978; CORDEEVA 1973; ZIMMERMAN ET AL., 1965a,b KORSRUD ET AL., 1973  CORNISH ET AL., 1970 PELLING ET AL., 1976 CLAMPITT, 1978  KORSRUD ET AL., 1973	THE LDH ISOCENZYMES ARE MOST USEFUL IN DISTINGUISHING DAMAGE IN OTHER ORGANS FROM DAMAGE IN THE LIVER. LDH HAS FIVE ISOENZYMES. LDH 1 IS RELATIVELY EASY TO QUANTITATE; THE OTHER FOUR ARE DIFFICULT TO SEPARATE AND QUANTITATE.
	RABBIT	CARBON TETRACHLORIDE	DINMAN ET AL., 1962; FOX ET AL., 1962	

TABLE C-1.6  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
MALATE DEHYDROGENASE (MD, EC 1.1.1.37)	BAT	CARBON TETRACHLORIDE DIETHANOLAMINE DIMETHYLNITROSAMINE THIOACETAMIDE	KORSRUD ET AL., 1972; REES AND SINHA, 1960; ZIMMERMAN ET AL., 1964; <sup>a,b</sup> KORSRUD ET AL., 1973 KORSRUD ET AL., 1973; REES AND SINHA, 1960	MD IS PRESENT IN THE GREATEST QUANTITIES IN THE MITOCHONDRIA. ITS RELEASE AFTER LIVER CELL DAMAGE IS SLOW AND PROLONGED.
	RABBIT	CARBON TETRACHLORIDE	DINMAN ET AL., 1962; FOX ET AL., 1962.	

TABLE C-1.7  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
GUINEA PIG	CARBON TETRACHLORIDE DIFLUOROMETHANE DIFLUOROMETHANESULFAMIC PHENOLISOCACLOUTINS THIOPENTONE SODIUM	JUDAH ET AL., 1963; REES AND SINHA, 1960; ZIMMERMAN ET AL., 1965a,b KORSKUD ET AL., 1973 IKEGAMI AND BASSIR, 1977 JUDAH ET AL., 1963; KORSKUD ET AL., 1973; REES AND SINHA, 1960	LCO ACTIVITIES ARE HIGH IN ACUTE HEPATIC NECROSIS AND ACUTE VIVAL HEPATITIS.
RABBIT	CARBON TETRACHLORIDE	DINNAN ET AL., 1962; FOX ET AL., 1962	
CHICKEN	CHLOROWANYCHYLIC ACID	SUZUKI ET AL., 1977	

TABLE C-1.8  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SERUM CHOLINESTERASE (ChE, EC 3.1.1.7)	RATS, RABBITS, MONKEYS	CADMUM, CARBONATE/CHLORIDE, HALOTHANE, SODIUM SELENATE	CUTLER, 1974; HEYWOOD ET AL., 1978; LEININGER ET AL., 1974; NOMIYAMA ET AL., 1979; SHUTLA AND SHAstry, 1975	ChE IS REFERRED TO AS "PSEUDOCHELOLINESTERASE" TO DISTINGUISH IT FROM ACHE FOUND IN ERYTHROCYTES AND NERVE CELLS. ACTIVITIES OF ChE FALL DURING PARENCHYMATOUS LIVER DYSFUNCTION. IT IS NOT A MORE SENSITIVE INDEX OF PARENCHYMAL FUNCTION THAN SOME OTHER ENZYMES DESCRIBED IN THIS REPORT.

TABLE C-1.9  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
ALDOLASE (ALD, EC 4.1.2.7 or EC 4.1.2.5)	RAT	CARBON TETRACHLORIDE DIETHANOLAMINE DIMETHYLNITROSAMINE THIOACETAMIDE	KORSJUD ET AL., 1972 KORSJUD ET AL., 1973	MARKE INCREASES IN ALD SERUM ACTIVITY ARE OBSERVED IN LABORATORY ANIMALS WITH HEPATIC NECROSIS.
	RAT	CARBON TETRACHLORIDE	ZIMMERMAN ET AL., 1965a,b	
	RABBIT	CARBON TETRACHLORIDE	DINMAN ET AL., 1962; FOX ET AL., 1962	

TABLE C-1.10  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
PHOSPHOHEXOKINASE (EC 5.3.1.9)	RAT	CARBON TETRACHLORIDE	ZIMMERMAN ET AL., 1965a,b	MARRED INCREASES IN PHK SERUM ACTIVITY ARE OBSERVED IN LABORATORY ANIMALS WITH HEPATIC NECROSIS.
	RABBIT	CARBON TETRACHLORIDE	DINMAN ET AL., 1962; FOX ET AL., 1962	

TABLE C-1.11  
BIOCHEMICAL DAMAGE INDICATORS: SERUM ENZYME ACTIVITY

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
TRIUSINE AMINopeptidase (LAP, EC 3.4.1.1)	RAT	BILE DUCT LIGATION CARBON TETRACHLORIDE OROTIC ACID SODIUM PHENOBARBITONE	IDEO ET AL., 1972 CLAMPITT, 1978; IDEO ET AL., 1972 CLAMPITT, 1978	LAP SERUM ACTIVITY RISES FROM HEPATOBILARY DISEASES AND IT IS MOST USEFUL IN DETECTING OBSTRUCTIVE BILARY CONDITIONS.
	RABBIT	CARBON TETRACHLORIDE	DINMAN ET AL., 1962	
	DOC	CHLOROFORM	KEEFE ET AL., 1978	
	SQUIRREL MONKEY	CHENODEOXYCHOLIC ACID	SUZUKI ET AL., 1977	
	RHECUS MONKEY	CHENODEOXYCHOLIC ACID URSODEOXYCHOLIC ACID	FEDOROWSKI ET AL., 1978	

TABLE C-2: CARBOHYDRATE METABOLISM  
MEASUREMENTS OF HEPATIC FUNCTION:

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
GLUCOSE	RAT	CALCIUM CHLORIDE XYLITOL	KOTSONIS AND KLAASEN, 1977 TRIPHAUT ET AL., 1977	PLASMA GLUCOSE LEVELS ARE HIGHLY VARIABLE IN SMALL LABORATORY ANIMALS AND ARE AFFECTED BY A NUMBER OF FACTORS IN ADDITION TO LIVER DISFUNCTION.
	RHECUS MONKEY	CHENODEOXYCHOLIC ACID URSODEOXYCHOLIC ACID	FEDOROWSKI ET AL., 1978	

TABLE C-3  
MEASUREMENTS OF HEPATIC FUNCTION: LIPID METABOLISM

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
CHOLESTEROL.	RAT	B-GLYCEROPHOSPHATE CARBON TETRACHLORIDE ETHANOL OROTIC ACID PHENOBARBITAL PHOSPHORUS POLYCHLORINATED BIPHENYL PRASEODYMIUM NITRATE XYLITOL	RAHEJA ET AL., 1977 CLAMPITT, 1978 SEAKINS AND ROBINSON, 1964 CLAMPITT ET AL., 1978 SEAKINS AND ROBINSON, 1964 ALLEN ET AL., 1974 VON LEHMANN ET AL., 1976 TRUHAUT ET AL., 1977	NORMAL CHOLESTEROL LEVELS ARE LOW IN RATS AND VARY SUFFICIENTLY TO HAVE ONLY LIMITED USEFULNESS IN TOXICITY STUDIES.
LIPIDS, TOTAL	RAT	PRASEODYMIUM NITRATE XYLITOL	VON LEHMANN ET AL., 1976 TRUHAUT ET AL., 1977	
RHECUS MONKEY		POLYCHLORINATED BIPHENYL	ALLEN ET AL., 1974	
LIPOPROTEIN	RAT	PRASEODYMIUM NITRATE	GRAJENSKI ET AL., 1975 VON LEHMANN ET AL., 1976	
PHOSPHOLIPIDS	RAT	B-GLYCEROPHOSPHATE ETHANOL PHOSPHORUS PRASEODYMIUM NITRATE	RAHEJA ET AL., 1977 SEAKINS AND ROBINSON, 1964 SEAKINS AND ROBINSON, 1964 GRAJENSKI ET AL., 1975 VON LEHMANN ET AL., 1976	

TABLE C-4  
MEASUREMENTS OF HEPATIC FUNCTION: PROTEIN METABOLISM

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
PROTEIN (ALBUMIN, GLOBULIN)	RAT	ASPIRIN CARBON TETRACHLORIDE	BALASUBRAMANIAN ET AL., 1977 BALASUBRAMANIAN ET AL., 1977; CLAMPITT, 1978; CUTLER, 1974	DECREASES IN LIVER PROTEIN SYNTHESIS MAY BE CAUSED BY A NUMBER OF FACTORS INCLUDING MALNUTRITION, LIVER DISEASE, AND VARIOUS DRUGS.
		ETHANOL $\delta$ -HEPTACHLOROCYCLOHEXANE	BALASUBRAMANIAN ET AL., 1977 CLAMPITT, 1978 GRAJEMSKI ET AL., 1975 CLAMPITT, 1978 CUTLER, 1974	THE MONITORING OF PROTEIN SYNTHESIS IN LABORATORY ANIMALS IS NOT CONSIDERED USEFUL ROUTINE TOXICITY SCREENING.
	Rhesus MONKEY	CHENODEOXYCHOLIC ACID URSODEOXYCHOLIC ACID POLYCHLORINATED BIPHENYL	FEDOROWSKI ET AL., 1978 ALLEN ET AL., 1974	
THIOL TURBIDITY	MOUSE	ALLYL FORMATE, CARBON TETRA- CHLORIDE, HEPATIC AUTOLYSATE, PHOSPHORUS, <i>S. ENTERITIDIS</i>	CASALS AND OLITSKY, 1946	
FLOCCULATION	MOUSE	ALLYL FARNATE, CARBON TETRA- CHLORIDE, HEPATIC AUTOLYSATE, PHOSPHORUS, <i>S. ENTERITIDIS</i>	CASALS AND OLITSKY, 1946	
	GUINEA PIG	AMPHETAMINE SULFATE, PHENO- BARBITAL, RESERPINE	DECIAUCOMO ET AL., 1977	

TABLE C-4 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
FLOCCULATION (CONCLUDED)	RAT	CARBON TETRACHLORIDE SODIUM SELENATE	CUTLER, 1974	

TABLE C-5  
MEASUREMENTS OF HEPATIC FUNCTION: XENOBIOTIC METABOLISM

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
HEXOBARBITAL SLEEPING TIME	MOUSE	DIETHYL MALEATE PARAQUAT SELENIUM DEFICIENCY	CAGEN AND GIBSON, 1977	PROLONGATION OF BARBITURATE SLEEPING TIME IS A USEFUL TECHNIQUE FOR THE DETECTION OF HEPATIC INJURY.
	RAT	ACTINOMYCIN D ANABOLIC STEROIDS CARBON TETRACHLORIDE DIMETHYLSUFOXIDE HYDRAZINE MALEIC HYDRAZIDE	BADER ET AL., 1974 KULCSAR-CERGELY ET AL., 1975 MANCINI AND KOCSTS, 1974 AKIN AND NORRED, 1978	
PENTOBARBITAL SLEEPING TIME	MOUSE	$\alpha$ -NAPHTHYLISOTHIOCYANATE CONGENERS	BECKER AND PLA, 1965	
	RAT	KEPONE NITROSAMINES	MENENDALE ET AL., 1978 NISHIE, 1978	
ZOXAZOLAMINE PARALYSIS (DURATION)	RAT	BENZOPYRENE LEAD ACETATE PHENOBARBITAL	CHOW AND CORNISH, 1976	
SODIUM BENZOATE/BENZOIC ACID DETOXIFICATION	RAT	CARBON TETRACHLORIDE SODIUM SELENATE	CUTLER, 1974	DECREASED URINARY HIPPURIC ACID EXCRETION IS A SENSITIVE INDICATION OF HEPATIC DAMAGE IN RATS. ACCURATELY TIMED URINE SAMPLES MUST BE OBTAINED WHEN THIS TEST IS USED. THIS MAY BE SUFFICIENTLY DIFFICULT IN SMALL

TABLE C- 5 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
SODIUM BENZOATE/BENZOIC ACID DETOXIFICATION	RAT (CONTINUED)	CARBON TETRACHLORIDE SODIUM SELENATE	CUTTLER 1974	LABORATORY ANIMALS TO LIMIT ITS ROUTINE USE IN SCREENING PROGRAMS.

APPENDIX D

MEASUREMENT OF HEPATIC DAMAGE:  
IN VITRO TECHNIQUES

TABLE D-1  
MEASUREMENT OF HEPATIC DAMAGE: IN VITRO TECHNIQUES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
LIVER SLICES	RATS, RABBITS	PARACETAMOL, PHENOTHIAZINES	ODDOWNE ET AL., 1968; MCLEAN AND NUTTALL, 1978	THIS METHOD IS USED FOR RESEARCH PURPOSES AND IS NOT A ROUTINE SCREENING PROCEDURE.

TABLE D-1.1  
MEASUREMENT OF HEPATIC DAMAGE: IN VITRO TECHNIQUES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
PERFUSION TECHNIQUES	RATS	DANTHOLENE, SODIUM, DDT, P,P'-DDT, HEKACHLOROBENZENE, HEXACHLOROCYCLOBENZENE, KEPONE, LEAD, MIREX, PCB, TOXAPHENE	ABERNATHY ET AL., 1978; BUCHANAN AND FILKINS, 1976; MEHENDALE, 1976a & b, 1977a, b & c, 1978; MEHENDALE ET AL., 1977	MOST PERfusion TECHNIQUES REQUIRE CONSIDERABLE SKILL AND EQUIPMENT. THESE TECHNIQUES ARE PRINCIPALLY USED FOR RESEARCH PURPOSES.

TABLE D-1.2  
MEASUREMENT OF HEPATIC DAMAGE: IN VITRO TECHNIQUES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
HEPATOCYTE CULTURES	MOUSE, RATS, HUMAN (CHANG)	ACETAMINOPHEN, ANOBARBITAL, ANESTHETICS, TRICYCLIC ANTI- DEPRESSANTS, ANILINE, BENZO(A)PYRENE, CHLORO- BIPHENYL, CHLOROFORM, CHLORO- PROPRAMAZINE, CHLOROPROMAZINE, METABOLITES, DEXMETHASONE, DIOLYL SODIUM (OXYPHENISATIN BASE), ENDOTOXIN, ENFLURANE, ERYTHROMYCIN (BASE CETYL, SULFATE, ESTOLATE, STEARATE), ETHANOL, ETHER, FORMALDEHYDE, FORMIC ACID, HALOTHANE, HYDRO- CORTISONE, HYDROGEN PEROXIDE, ISOFLURANE, LIDOCaine, METHoxy FLURANE, METHOHEXITAL, METHORY FLURANE, METHYLCLO LANTHENE, NITRO- FLURANTIN, NORETHIN DRONE, ACETATE, O,P'-DDT, PARAVARINE, PHALLODIN, PROMAZINE, SALICY- LATES, SODIUM LAURYL SODIUM SALICYLATE, SULFORBOMPHTHALEIN, SULFOSUCCINATE, TETRACYCLINE HYDROCHLORIDE, THIOPENTAL, THIOKANTHENE, TRICYCLIC ANTI-DEPRESSANTS	ABERNATHY AND ZIMMERMAN, 1975; ABERNATHY ET AL., 1975; ABERNATHY ET AL., 1977; AGOSTA ET AL., 1978; BAUR ET AL., 1975; BERRY AND FRIEND, 1968; BISSELL AND GUZELIAN, 1975; BISSELL AND HAMAKER, 1976; BONNEY ET AL., 1974; CORSEN ET AL., 1966; DECAD ET AL., 1977; DJOUZE, 1975; DUJOVNE AND SHOBMAN, 1970; DUJOVNE ET AL., 1970, 1972; FRY AND BRIDGES, 1979; GRISHAM ET AL., 1978; GOTO ET AL., 1976; GUZELIAN AND BISSELL, 1976; GUZELIAN ET AL., 1977; HERBERT ET AL., 1971; JACKSON, 1971; JONES ET AL., 1978a, 1978b; KROKER AND HEGNER, 1973; LAISHE AND WILLIAMS, 1976a, 1976b MICHALOPOULOS ET AL., 1976; PARTIZA ET AL., 1975; SAJAH ET AL., 1978; SCHMELTZ ET AL., 1978; SEGLIN, 1972; STACEY ET AL., 1978; STEGE ET AL., 1975; STORY AND FREEDLAND, 1978; TOLMAN ET AL., 1978; VAN BEEZOOIJEN ET AL., 1976; WIEBKIN ET AL., 1978; YASHIKAWA ET AL., 1978; ZAHLEN AND STRATHMAN, 1974; ZEFFERT AND PAUL, 1972; ZIMMERMAN AND KENDLER, 1970;	FRESHLY ISOLATED HEPATO- CYTES SUSPENSIONS PROVIDE A USEFUL MODEL SYSTEM FOR SHORT-TERM TOXICITY STUDIES. VIABLE HEPATO- CYTE SUSPENSIONS ARE RELATIVELY EASY TO PREPARE AND USE IN ASSESSING TOXICITY. HOWEVER, THEY MUST BE USED SOON AFTER ISOLATION, USUALLY WITHIN A FEW HOURS, OR THEY BEGIN TO DETERIORATE. A LARGE NUMBER OF SUBSTANCES CAN BE ASSESSED USING A SINGLE CELL POPULATION.  MONOLAYER HEPATOCYTE SUSPENSIONS MAY BE ALSO USED IN SHORT-TERM SCREENING; HOWEVER, THEY ARE MORE DIFFICULT TO MAINTAIN AND TREAT THAN SUSPENSIONS. DIFFEREN- TIATION MAY BECOME A PROBLEM AFTER CELLS ARE ESTABLISHED IN CULTURE AND MAY BE AVOIDED BY SPECIALIZED CULTURING TECHNIQUES.

TABLE D-1, 2 (CONCLUDED)

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
HEPATOCYTE CULTURES			ZIMMERMAN ET AL., 1973; ZIMMERMAN ET AL., 1974	

TABLE D-1.3  
MEASUREMENT OF HEPATIC DAMAGE: IN VITRO TECHNIQUES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
TISSUE HOMOGENATES	MICE, RATS, GUINEA PIGS, RABBITS, MINNOWS, CHICKEN, DOGS	ACRYLONITRILE, ALLYL ALCOHOL, $\alpha$ -AMINOBENZENE, BROMOBENZENE, CANABIS, CARBON TETRACHLORIDE, CHLOROFORM, COCAINE, CYSTEINE, 1,1-DICHLOROETHYLENE, DIBUTYLHALOATE, DIMETHYL-NITROSAMINE, N,N'-DIPHENYL-PHENYLENDIAMINE, ETHANOL, ETHANOL PHOSPHORUS, FATTY ACIDS, GLUTATHIONE, $\beta$ -GLYCEROPHOSPHATE, LANTHANUM TRICHLORIDE, LEAD ACETATE, METHYLCHOLANTHRENE, PARACETAMOL, PHALLOIDIN, PHENOBARBITAL, THIGSPOROUS, PHYTOHEMAGGLUTININS, PLASTICIZERS AND ANALOGS, POLYBONIUM-4-BIPOVINT, PRASODIUM, PROPYL GALLATE, TETRAZOLE, THIOACETATIDE, TRICHLOROPROPANE OXIDE, VINYL CHLORIDE, VINYLIDENE CHLORIDE	ASADA, 1958; ASADA AND CALAMBOS, 1963; CONOLY AND JAEGER, 1978; CORNISH BLOCK, 1960; DIANZANI, 1957; DINNAN ET AL., 1962; EBNER AND COURI, 1979; EVANS AND HARBISON, 1976; FUJISAWA ET AL., 1976; CORDEEVA, 1973; HANZLIK ET AL., 1978; IKEUCHI AND BASSIR, 1977; JIMAS ET AL., 1978; JAEGER ET AL., 1977; JUGGI, 1977; KADAS ET AL., 1974; KEFTE ET AL., 1978; KUMATA ET AL., 1975; MAXWELL ET AL., 1973; MCKENNA ET AL., 1977; MCLEAN AND NOTTALL, 1978; MOORE ET AL., 1978; PANI ET AL., 1972; RAHEJA ET AL., 1977; REES AND SINHA, 1960; SEAKINS AND ROBINSON, 1964; STRUBELT ET AL., 1978; TREJO ET AL., 1972; VERSCHUREN ET AL., 1973; WATANABE ET AL., 1978; WILLSON AND HART, 1977a & b; WITSCHI AND SAINT-FRANCOIS, 1972	THESE PREPARATIONS HAVE BEEN USED PRINCIPALLY IN METABOLISM STUDIES. THEY ARE NOT CONSIDERED USEFUL FOR ROUTINE TOXICITY SCREENING.

TABLE D-1.4  
MEASUREMENT OF HEPATIC DAMAGE: IN VITRO TECHNIQUES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
ISOLATED MITOCHONDRIA	RATS, RABBITS	ACTINOMYCIN D, CC14, CARBON TETRACHLORIDE, COBALT CHLORIDE, CYCLOHEXIMIDE, DIMETHYL-NITROSAMINE, ETANOL, ETHIONINE, FATTY ACIDS, KERONE, PHOSPHORUS, POLY-CHLORINATED BIPHENYL, SODIUM ARSENATE, 2,3,7,8-TETRACHLOROBIPHENOL-p-DIOXIN, THIOACETAMIDE	DIANZANI, 1957; DIANZANI AND MARINARI, 1961; DINMAN ET AL., 1962; EBNER AND COULI, 1979; FUJISAWA ET AL., 1976; KITCHEN AND WOODS, 1978; REES AND SINHA, 1960; WOODS AND FOWLER, 1978	THESE ISOLATED ORGANELLE PREPARATIONS ARE ONLY USED IN RESEARCH APPLICATIONS.

TABLE D-1.5  
MEASUREMENT OF HEPATIC DAMAGE: IN VITRO TECHNIQUES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
ISOLATED ENDOPLASMIC RETICULUM	RATS	PHENOBARBITAL	ILYAS ET AL., 1978	THESE ISOLATED ORGANELLE PREPARATIONS ARE ONLY USED IN RESEARCH APPLICATIONS.

TABLE D-1.6  
MEASUREMENT OF HEPATIC DAMAGE: IN VITRO TECHNIQUES

TESTS EMPLOYED	SPECIES OF ANIMALS USED	SUBSTANCES TESTED	REFERENCES	COMMENTS
MICROSOMAL PREPARATIONS	MMCE, RATS, RABBITS, DOGS	ACETONE, ACTINOMYCIN D, AROCLOR 1224, BENZOPYRENE, CAPTION CHLORIDE, CARBON TETRACHLORIDE, COBALT CHLORIDE, CYCLOCHEXIMIDE, O,P'-DDT, DIMETHYLNITROSAMINE, ETHANOL, DITHIOCARBAMATES, ETHANOL, HEXABROMOBIPHENYL, HYDRAZINE, KERONE, LEAD ACETATE, MALEIC HYDRAZIDE, 3-METHYLCOLANTHRENE, MIREX, PARACETAMOL, PHENOBARBITAL, PHOSPHORUS, POLYBROMINATED BIPHENYL, POLICHLORINATED BIPHENYL, PRASODIUM NITRATE, SODIUM ARSENATE, 2,3,7,8-TETRACHLORO-DIBENZO-p-DIOXIN, THIOPRAN DISULFIDE	AKIN AND NORRED, 1976; ALLEN ET AL., 1976; BADER ET AL., 1976; CHOW AND CORNISH, 1978; COPELAND AND CRAMER, 1974; CORNISH AND BLOCK, 1960; DINMAN ET AL., 1962; FUJISAWA ET AL., 1976; HORIUCHI ET AL., 1978; ILYAS ET AL., 1978; KAMINSKY ET AL., 1978; KITCHEN AND WOODS, 1978; YOTSONS AND KLAASSEN, 1977; MARKELL ET AL., 1973; MCCORMACK ET AL., 1978; MEHENDALE ET AL., 1973, 1977; PANI ET AL., 1972; RAMBOER ET AL., 1972; SIFES ET AL., 1978; VON LEBHANN ET AL., 1976; WILSON AND HART, 1977 a & b; WOODS AND FOWLER, 1978; ZEMAITIS AND GREEN, 1979	THESE ARE COMMON PREPARATIONS WHICH ARE USED IN METABOLISM STUDIES OR IN OTHER STUDIES WHERE METABOLIC INDUCTION IS NECESSARY. THEY ARE NORMALLY NOT USED SEPARATELY AS MODEL SYSTEMS IN TOXICITY STUDIES, BUT ARE USED IN COMBINATION WITH OTHER MODELS SYSTEMS SUCH AS CULTURED CELLS.

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